Effects of phosphite on disease development and histological responses in *Eucalyptus marginata* infected with *Phytophthora cinnamomi*

by

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Declaration

I declare that this thesis is my own account of my research and contains as its main content, work which has not previously been submitted for a degree at any tertiary education institution.

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Abstract

Phosphite is currently used for the management of *Phytophthora cinnamomi* in native plant communities. A greater understanding of how phosphite affects the host-pathogen interaction is required in order to determine the most effective treatment. This thesis aimed to investigate the effects of applied phosphite concentration on phytotoxicity, *in planta* concentration of phosphite, disease development and anatomical responses of *Eucalyptus marginata*.

Spraying the foliage to run-off with 7.5 and 10 g phosphite/L led to the development of severe leaf necrosis within 7 days, with greater than 60% of the leaf area damaged. Moderate phytotoxicity was observed after treatment with 5 g phosphite/L. *In planta* concentration of phosphite in stems, lignotubers and roots did not differ significantly between applied concentrations of phosphite. Stem tissue contained the largest concentration of phosphite at one week after spraying, with approximately 210 and 420 µg phosphite/g dry weight detected after treatment with 5 and 10 g phosphite/L, respectively.

In a subsequent field trial, the applied concentration of phosphite was found to affect the duration of effectiveness of phosphite in protecting *E. marginata* seedlings from stem colonisation by *P. cinnamomi*. Plants were wound-inoculated with *P. cinnamomi* at 6-monthly intervals after spraying with phosphite. The 2.5 and 5 g phosphite/L treatments were effective against colonisation by *P. cinnamomi* when inoculated 0 and 6 months after spraying, but only the 5 g phosphite/L treatment inhibited *P. cinnamomi* within 12 months of spraying. Phosphite had no effect on colonisation by *P. cinnamomi* when plants were inoculated at 17 months after spraying. The *in planta* concentration of phosphite detected in the leaves, stems and roots of plants treated with 5 g phosphite/L did not differ significantly between the time of harvest or tissue type at 0.2 and 6 months after spraying. *P. cinnamomi* remained viable in plants treated with phosphite.

Treatment with 2.5 and 5 g phosphite/L when *P. cinnamomi* was well-established in the stems was ineffective at preventing the death of *E. marginata*. Between 45 and 89% of plants were girdled on the day of spraying. Spraying plants with 2.5 and 5 g phosphite/L when conditions were less favourable for the pathogen reduced the mortality of *E. marginata* for up to 10 months.

E. marginata seedlings responded to damage by *P. cinnamomi* with the production of kino veins and woundwood. Bark lesions were in the process of being sloughed off by 7 months after inoculation in plants that remained alive.

In plants of a resistant (RR) clonal line and susceptible (SS) clonal line, phosphite treatment inhibited lesion extension in stems, but lesions did not indicate the amount of stem colonised by *P. cinnamomi*. The pathogen was isolated from up to 17 cm beyond the lesion front in the RR clonal line. Treatments that reduced the mortality of *E. marginata* were 5 g phosphite/L in the RR clonal line (RR/5) and 10 g phosphite/L in the SS clonal line (SS/10).

Uninoculated plants were wounded with liquid nitrogen to determine the microscopic responses to injury in the absence of the pathogen. Wound closure was achieved within 21 days of wounding, with callus formation and vascular cambium regeneration. A wound periderm separated wounded tissue from healthy tissue, adjacent to a lignified boundary zone. Two types of phellem were observed – thin-walled phellem (TnP) and thick-walled phellem (TkP). The first-formed TnP layers contained variable-shaped cells, while subsequent layers were more cubical in shape. Multiple TnP layers developed up to 42 days after wounding, with TkP cells sandwiched between the TnP layers. Genotype and phosphite treatment did not affect the wound responses.

Inoculated plants with a restricted lesion extension also formed a wound periderm to separate damaged tissue from healthy tissue. Phosphite treatment stimulated the responses to *P. cinnamomi* in both clonal lines. Early development of the wound periderm was visible by 6 days after phosphite treatment. It was

preceded by the formation of a ligno-suberised boundary zone in the cambial zone and in phloem parenchyma cells existing prior to injury. Suberin was not detected in the SS/0 treatment. TnP layers completely surrounded lesioned tissue in plants still alive by 24 days after phosphite treatment. Extensive callus production was evident in the SS/10, RR/5 and RR/10 treatments.

Temperature affected the post-inoculation efficacy of phosphite and anatomical responses of *E. marginata*. At 20°C, lesion extension was restricted in both clonal lines of *E. marginata*, irrespective of phosphite treatment. Greater than 70% of inoculated plants in all treatments produced a ligno-suberised boundary zone at 20°C and between 30 and 70% formed a wound periderm. At 28°C, lesion extension was reduced in phosphite-treated plants at 7 days after treatment. However, lesions continued to extend up to 5 mm per day in the SS clonal line and very few SS plants formed a wound periderm at the lesion front. This contrasted with the strong responses to abiotic wounding observed in uninoculated SS plants at 28°C. The most extensive responses to *P. cinnamomi* were detected in the RR/5 treatment at 28°C, with a ligno-suberised boundary zone and differentiated TnP of a wound periderm observed in greater than 70% of plants. This treatment resulted in significantly less girdled plants than all other treatments at 28°C, including the RR/0 treatment. At 23 and 24°C, there was no significant difference in acropetal lesion extension or circumferential lesion spread between clonal lines. The inoculation technique and environmental conditions may have resulted in too high a disease pressure for a full expression of resistance in the RR clonal line.

This thesis demonstrates that phosphite has the potential to enhance the resistance of young *E. marginata* and enable them to survive infection by *P. cinnamomi*. However, its effectiveness is dependent upon a number of factors, including host resistance, environmental conditions, the applied phosphite concentration and the timing of application.

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Chapter 1: Introduction and literature review

1.1 General introduction

In Australia, plant pathogens of the genus *Phytophthora* are responsible for widespread losses in pastoral, agricultural, horticultural, ornamental and forest industries (Irwin *et al*., 1995). *P. cinnamomi* Rands is considered to be one of the most destructive pathogens in natural ecosystems (Cowling and Wills, 1994; Weste 1994), causing root and collar rot in an exceptionally wide host range (Colquhoun and Hardy, 2000). The pathogen is a water mould (class Oomycetes), and reproduces asexually by zoospores and chlamydospores, and sexually by oospores. Propagules of *P. cinnamomi* spread into previously uninfected areas by the movement of infected soil and water, either autonomously or with the help of vectors, such as animals.

Early European settlers are considered likely to be responsible for the introduction of *P. cinnamomi* to Australia (Podger *et al*., 1996). In Western Australia, the disease caused by *P. cinnamomi*, commonly referred to as “jarrah dieback”, was first noticed in the native forest in 1921 (Podger, 1972). Since then, *P. cinnamomi* has had a major impact on the jarrah (*Eucalyptus marginata* Donn ex Sm.) forest in the south-west of Western Australia, where many of the plant taxa are susceptible to *P. cinnamomi* (Shearer and Dillon, 1995). Changes occur in the plant community structure after infestation with *P. cinnamomi*, with the death of susceptible species and greater abundance of resistant species (Wills and Keighery, 1994). The impact of *P. cinnamomi* on biodiversity in Western Australia is now recognised as a “biological disaster of global significance” (Podger *et al*., 1996).

Recent efforts to reduce the impact of *P. cinnamomi* in native communities have focussed on the use of the systemic chemical phosphite (phosphonate). Phosphite is currently used to protect rare and endangered plant species and rare

native ecosystems (Hardy *et al*., 2001). Alcoa World Alumina Australia is developing protocols for the treatment of native plant communities in and adjacent to mining areas (Colquhoun and Hardy, 2000). In order to determine the most effective treatment, a greater understanding of how phosphite affects the host-pathogen interaction is required. The following literature review discusses plant resistance mechanisms and other factors that influence host-pathogen interactions. It then outlines current understanding of the role of phosphite in enhancing plant resistance in response to pathogen invasion.

1.2 What is plant resistance?

Plants are often described as being either susceptible or resistant to a particular pathogen, although in reality the two are extremes on a continuum (Guest and Brown, 1997a). The gradient in plant resistance was clearly demonstrated by Cahill *et al*. (1989) in a study of histological changes induced by *P. cinnamomi*. Susceptibility of plants is defined as the relative inability of an organism to impede attack by a parasite, while resistance is the ability of hosts to hinder growth and activity of a parasite (Bos and Parlevliet, 1995). As discussed by Erwin and Ribeiro (1996), there are different types of plant resistance, and the terminology in the literature for these lacks uniformity. Briefly, there are two major groupings of resistance: vertical resistance (also known as host-specific, race-specific and true resistance), where disease does not occur; and horizontal resistance (or general, rate-reducing or field resistance), where the rate of disease development is usually slower than on a susceptible plant (Erwin and Ribeiro, 1996). Resistance can occur at different stages of the host-pathogen interaction. Plant defences may prevent initial germination and penetration by the pathogen, or may restrict colonisation and reproduction once the pathogen is established within the plant (Parlevliet, 1979).

1.3 Plant resistance mechanisms

Resistance mechanisms can be divided into two components – pre-existing/passive defences and induced/active defences. In order to enter a host, the pathogen must first overcome pre-existing barriers such as the cuticle and suberised periderm (Kolattukudy, 1985) and fungitoxic substances, such as phenolic compounds (Agrios, 1997). Once the pathogen is recognised by the plant, active defence responses begin. These can be further divided into two categories – rapid and delayed active defences. Some of the rapid responses that inhibit pathogen development include the oxidative burst (Guest and Brown, 1997a), the hypersensitive reaction (Goodman and Novacky, 1994) and the production and accumulation of phytoalexins (Barz *et al*., 1990; Kuc, 1995; Smith, 1996). Containment of the pathogen and wound repair are delayed defences, and usually involve anatomical changes at the junction of healthy and damaged tissues. These will now be discussed in more detail.

1.3.1 Anatomical responses to bark injury

There is an extensive range of literature that describes the responses of bark to injury. Papers by Mullick (1977), Biggs *et al*. (1984) and Biggs (1992) provide the most comprehensive descriptions. However, before the responses to injury can be fully understood, it is essential to have a basic understanding of the anatomy of bark.

Where possible, the terminology for bark anatomy used in this thesis follows the suggestions of Trockenbrodt (1990). In woody plants, the term bark refers to all tissue external to the vascular cambium (Srivastava, 1964). This may include secondary phloem produced by the vascular cambium, any remaining primary phloem and cortex of primary origin, periderm/s and epidermis (Trockenbrodt, 1990).

A periderm consists of three parts: the phellogen, phellem and phelloderm. The phellogen is a meristematic layer of parenchyma cells, and produces phellem (usually with suberised walls) to the outside and phelloderm to the inside. Two types of phellem may be produced by the phellogen – thin- (TnP) and thick-walled (TkP) phellem (Bramble, 1936; Chattaway, 1953; Martin and Crist, 1970; Grozdits *et al*., 1982; Godkin *et al*., 1983).

The periderm that replaces the epidermis as a protective layer is commonly called the first periderm (Srivastava, 1964; Fahn, 1982). Expansion of the stem or root may rupture the first periderm and a sequent periderm forms deeper in the bark (Chattaway, 1953). In most plants, progressively deeper sequent periderm layers form in the bark (Srivastava, 1964). All tissues external to the phellogen of the most recently formed periderm die and are referred to as rhytidome (Srivastava, 1964). This dead tissue is eventually shed. The first and sequent periderms that are part of the process of secondary growth have traditionally been called 'normal' or 'natural' periderms (Bramble, 1936; Mullick, 1977; Trockenbrodt, 1994).

Plants respond to bark injury with the formation of a periderm, commonly referred to as a wound periderm (Bramble, 1936; Esau, 1965; Biggs *et al*., 1984; Bostock and Middleton, 1987; Trockenbrodt, 1994; Wier *et al*., 1996; Woodward and Pocock, 1996). According to Esau (1965), wound periderms are similar to natural periderms in their origin and growth, but differ in the timing of origin and occurrence next to injury. However, a study of the pigments in coniferous periderms led Mullick and Jensen (1973) to suggest that the terms natural and wound to distinguish between periderms may be misleading. They proposed the term exophylactic periderm (EP) for the tissue that protects living cells from the external environment. This included the first periderm (FEP) and sequent periderms containing the same pigments (SEP). The latter only developed adjacent to necrophylactic periderms and were associated with exfoliation of external dead and diseased tissue, thus

eventually exposing the SEP at the surface. The term necrophylactic periderm (NP) was proposed for all periderms that formed between dead and living cells, including wound periderms, normal sequent periderms and pathological periderms (Mullick and Jensen, 1973).

Subsequently, Mullick (1975) determined that the formation of non-suberised impervious tissue (NIT) was a pre-requisite for NP formation in conifers. The term NIT referred to cells existing prior to injury that subsequently became enlarged and lignified. The imperviousness of the lignified tissue was determined by the ferric chloride-potassium ferricyanide (F-F) test, which was developed by Mullick (1975). Work conducted by Soo (1977) confirmed that NIT was invariably present prior to NP formation in a number of conifer species. In summary, Mullick (1977) concluded that a shallow injury to bark leads to the formation of NIT, followed by phellogen restoration (NP formation) in healthy tissue adjacent to the NIT (Figure 1.1). Mullick (1977) also described the consequences of deeper wounding, but these will not be discussed here.

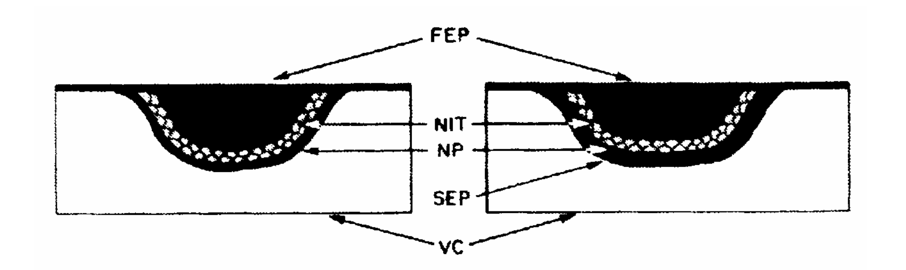


Figure 1.1: Schematic diagram of the response of bark to injury, using terminology proposed by Mullick and Jensen (1973). FEP=first exophylactic periderm, NIT=non-suberised impervious tissue, NP=necrophylactic periderm, SEP=sequent exophylactic periderm, VC=vascular cambium. Figure has been adapted from Puritch and Jensen (1980).

Biggs *et al.* (1983) reported that the response of poplars to *Cytospora chrysosperma* was similar to that described by Mullick (1977), with the formation of NIT and NP. However, with the use of more sophisticated tests for

suberin, Biggs later questioned the non-suberised nature of the impervious tissue (Biggs *et al*., 1984). A combination of histochemical tests and fluorescence microscopy revealed an intracellular suberin lining associated with impervious cells (Biggs, 1985a). Woodward and Pearce (1988) also observed that impervious tissue was suberised, and referred to it as suberised impervious tissue (SIT). Robinson (1997) reported that it was the cells caught between the NIT and the developing phellogen that became suberised, and not the impervious tissue itself. Despite the finding by Tippett and Hill (1984) that the imperviousness of tissue in *E. marginata* is directly related to suberisation, the use of the terms NIT or SIT to describe the responses of *E. marginata* will be avoided in this thesis.

Very few researchers have adopted the terminology proposed by Mullick and Jensen (1973) and Mullick (1975). Trockenbrodt (1990) suggests that the distinction made by Mullick and co-workers between periderms requires further scrutiny. In this thesis, the terms ligno-suberised boundary zone and wound periderm (Biggs, 1992; Spanos *et al*., 1999) will be used rather than NIT and NP.

1.3.2 Anatomical responses to vascular cambium and xylem injury

1.3.2.1 Wound closure

In wounds inflicted to the depth of the xylem, the cambial zone at the margin of the wound produces undifferentiated parenchymatous callus cells (Shigo, 1989). As the callus tissue extends to cover the wounded surface, a phellogen develops near the outer surface of the callus and a new vascular cambium (VC) forms deeper in the callus (Larson, 1994). Xylem and phloem elements produced by the regenerated VC are usually abnormal in appearance until the VC becomes more organised (Zimmermann and Brown, 1971). Wound closure is considered to be complete when the continuity of the VC is re-established (Armstrong *et al*, 1981).

1.3.2.2 Compartmentalisation

The CODIT (compartmentalisation of decay in trees) model has been proposed to describe the responses of xylem to injury (Shigo and Marx, 1977; Shigo, 1984). According to Shigo (1984), trees form boundaries that isolate injured tissues and resist the spread of pathogens. This compartmentalisation is a two-part process, with the formation of reaction zones and barrier zones. Reaction zones are formed in tissues that are present at the time of injury, while barrier zones are tissues formed by the living vascular cambium in response to injury (Shigo, 1984). Shigo and Marx (1977) named the boundaries 'walls', with reaction zones consisting of walls 1 to 3 (plugging of xylem vessels, cells around growth rings and rays), and barrier zones comprising wall 4, separating wood present at the time of injury from new wood. Wall 4 is considered to be the strongest (Shigo and Marx, 1977). Barrier zones are anatomically distinct from normal xylem, and often contain fewer and smaller xylem vessels (Mulhern *et al*., 1979; Smith, 1980; Shigo and Tippett, 1981; Rademacher *et al*., 1984; Lowerts *et al*., 1986; Wilkes, 1986). They are occasionally suberised (Pearce and Rutherford, 1981; Pearce and Holloway, 1984; Pearce and Woodward, 1986) and can contain phenolics (Tippett and Shigo, 1980; Pearce and Woodward, 1986; Tippett, 1986).

1.4 Interspecific variation in resistance to *P. cinnamomi*

Resistance to *P. cinnamomi*, where it occurs, is horizontal (Irwin *et al*., 1995). A number of Australian native plant species have been found to be resistant to *P. cinnamomi*. Studies using zoospores as the inoculum found that resistance did not occur at the penetration stage, since all species tested became infected with *P. cinnamomi*, regardless of their susceptibility (Halsall, 1978; Hinch and Weste, 1979; Weste and Cahill, 1982; Phillips and Weste, 1984; and Cahill *et al.*, 1989).

After infection, root growth initially ceased in both susceptible and resistant plants but subsequently resumed in the resistant species, enabling the plants to outgrow the pathogen attack (Phillips and Weste, 1984 and Cahill *et al.*, 1989).

In the interaction between the resistant *Acacia pulchella* and *P. cinnamomi*, the host cells immediately surrounding hyphae died rapidly, indicative of a hypersensitive reaction (Tippett and Malajczuk, 1979). No such response was evident in susceptible *Eucalyptus* spp. examined for similar changes (Tippett *et al*., 1977). Papillae were also observed to form close to invading hyphae in *A. pulchella* (Tippett and Malajczuk, 1979). The formation of papillae or callose deposits was found to be associated with the degree of resistance to *P. cinnamomi* in a study comparing the responses of 13 species to infection by *P. cinnamomi* (Cahill and Weste, 1983). However, there was no evidence that callose prevented pathogen growth (Cahill and Weste, 1983).

An examination of histological changes induced by *P. cinnamomi* in a number of species, ranging from fully susceptible to resistant to the pathogen, failed to find a specific feature that was consistently associated with resistance (Cahill *et al.*, 1989). Several defence responses (such as the lignification of cell walls, deposition of phenolics and the formation of papillae) were observed more often in the resistant species but also occurred in some susceptible species (Cahill *et al.*, 1989).

1.5 Intraspecific variation in resistance to *P. cinnamomi*

Rands noted in the original description of *P. cinnamomi* that there was a great difference in the susceptibility of cinnamon trees to canker development by *P. cinnamomi* in the same stand (Rands, 1922). Plants were observed to form a mechanical barrier of gum that separated lesioned material from healthy tissue. In susceptible plants, the spread of *P. cinnamomi* was only temporarily stopped at the

gum barrier, while in resistant plants, there were almost no outbreaks of *P. cinnamomi* from the gum barrier (Rands, 1922).

Screening for resistance to *P. cinnamomi* within species has identified resistant genotypes of *Persea americana* (Zentmyer, 1980), *Pinus radiata* (Butcher *et al*., 1984) and *E. marginata* (Stukely and Crane, 1994). Histological examination of *P. americana* roots after infection with *P. cinnamomi* showed that the moderately resistant genet (Duke 7) walled off infected tissues from uninfected tissues by the formation of wound periderm and whorls of cells surrounding infected phloem bundles (Phillips *et al*., 1987; Phillips, 1993). Wound periderm was never observed in the susceptible genet (Topa Topa) (Phillips, 1993).

Observations of *E. marginata* trees surviving in forest areas infested with *P. cinnamomi* led to an investigation of the genetic heritability of resistance in *E. marginata* (Stukely and Crane, 1994). The resistance of *E. marginata* to *P. cinnamomi* was determined to be under strong genetic control. With the discovery of genotypes resistant to *P. cinnamomi*, seedlings were propagated using tissue culture methods (McComb *et al*., 1990). Due to the expensive nature of micropropagating *E. marginata*, seed orchards have since been established to produce resistant plants for a lower cost (Colquhoun and Hardy, 2000).

Increased lignin and phenolic production was found to be associated with the resistance of clonal lines of 10-month-old *E. marginata* (Cahill *et al*., 1993). Within 48 h of inoculation with *P. cinnamomi*, the concentrations of lignin and phenolics were much higher than constitutive levels in the resistant lines. It was stressed, however, that other resistance mechanisms were likely to be involved (Cahill *et al*., 1993).

Barrier zones with associated kino veins formed in the interaction between *P. cinnamomi* and *E. marginata* trees (Tippett *et al*., 1983; Tippett and Hill, 1984; Davison *et al*., 1994). Kino vein formation is a general resistance mechanism to

pathogens (Tippett, 1986) and occurs in a number of *Eucalyptus* spp. (Jacobs, 1955). Kino within the veins contains polyphenols, and is often incorrectly referred to as gum (Skene, 1965). Tippett *et al*. (1983) concluded that kino vein formation in *E. marginata* was ineffective against *P. cinnamomi*, and the formation of wound and normal periderms was much more likely to be an important resistance mechanism in *E. marginata*. In infected roots of *E. marginata*, periderms formed around arrested lesions and provided an effective boundary to pathogen extension in 50% of the samples (Tippett *et al*., 1983). Wound periderm always preceded the development of a normal periderm, with the phellem of the latter being described as more ordered and cubical in transverse section than the former (Tippett and Hill, 1984).

1.6 Effect of environment on host-pathogen interactions

Seasonal differences have been observed in the resistance of plants to *P. cinnamomi*, and these can be attributed largely to the influence of temperature and moisture on the host-pathogen interaction (Tippett *et al*., 1989; Robin *et al*., 1994). With increasing temperatures up to 30°C, disease development was found to increase in severity in *E. marginata* (Shearer *et al*., 1987). Other studies report similar results in *E. marginata* (Grant and Byrt, 1984) and *P. americana* (Zentmyer, 1981). Growth of *P. cinnamomi in vivo* was restricted at temperatures above 30°C (Zentmyer, 1981; Shearer *et al*., 1987), which reflects the growth of *P. cinnamomi in vitro* above 30°C (Zentmyer *et al*., 1976; Phillips and Weste, 1985; Hüberli, 1995). However, as pointed out by Shearer *et al*. (1987), the outcome of host-pathogen interactions may also be determined by the effect of temperature on host responses, particularly when temperatures favourable for pathogen growth also favour host defence responses. The time taken for the completion of the wound response is inversely related to temperature in numerous plant species (Lipetz, 1970; Biggs and Northover, 1985; Biggs, 1986).

As reported in numerous articles (Schoeneweiss, 1975; Blaker and MacDonald, 1981; Vannini and Mugnozza, 1991), water stress can predispose plants to disease. Host responses may be inhibited by water stress, with delays observed in the formation of impervious tissue in firs subjected to dry conditions (Puritch and Mullick, 1975). However, water stress does not always give the pathogen an advantage over the host. The growth of *P. cinnamomi* is sensitive to water deficits in *E. marginata*, with the cessation of lesion extension in water-stressed plants despite optimal temperatures for pathogen growth (Tippett *et al*., 1987). By influencing how quickly the pathogen can grow and the rate at which the host can respond (Shearer *et al*., 1987; Tippett *et al*., 1987), the environment plays an important role in determining the extent of disease development in *E. marginata*.

1.7 Phosphite

1.7.1 What is phosphite?

Guest and Grant (1991) provides an extensive review of phosphite literature. Phosphite is a fungitoxic chemical that is used widely in the horticultural industry to control Oomycete diseases. It is reported to be effective against *Phytophthora* in numerous plant species, including avocado (Pegg *et al*., 1985; van der Merwe and Kotzé, 1994), cocoa (Holderness, 1990; Guest *et al*., 1994) and pineapples (Rohrbach and Schenck, 1985). As mentioned previously, it is now being used to protect native ecosystems in Western Australia from the disease caused by *P. cinnamomi* (Hardy *et al*., 2001).

Several terms are used in the literature to refer to the antifungal chemical, including phosphorous acid, phosphonic acid, phosphonate and phosphite. According to Boenig *et al*. (1991), the compounds used as fungicides are derived from phosphorous acid [$(OH)_3P$], which becomes phosphonic acid [$HPO(OH)_2$] in aqueous solution. To minimise phytotoxicity, a partially neutralised solution or salt

of phosphonic acid is used (Cohen and Coffey, 1986). According to Grant *et al*. (1990), the term phosphonate must be used to refer to the salts of phosphonic acid. However, Roos *et al*. (1999) reported that both the terms phosphonate and phosphite are acceptable according to IUPAC nomenclature. In this thesis, the term phosphite will be used to refer to the salt that is applied to the plants and the anion that is present in the plant. Reference will also be made to research conducted on the fungicide Fosetyl-Al, which is an alkyl phosphite and is ionised to phosphite *in planta*.

1.7.2 Direct antifungal activity of phosphite

In vitro tests have demonstrated that phosphite and Fosetyl-Al inhibit the growth and reproduction of *Phytophthora* spp. (Coffey and Bower, 1984; Fenn and Coffey, 1984; Coffey and Joseph, 1985; Dolan and Coffey, 1988; Ouimette and Coffey, 1989a; Jackson, 1997a; Komorek and Shearer, 1997; Wilkinson *et al*., 2001a). Reported EC_{50} values range from 1.3 – 224.4 µg/mL phosphite for the inhibition of mycelial growth, and from 0.1 – 44 µg/mL phosphite for the inhibition of sporangium formation and zoospore release. The fungitoxicity of phosphite has been found to vary within species (Coffey and Bower, 1984; Ouimette and Coffey, 1989a; Hüberli, 1994; Komorek and Shearer, 1997; Jackson, 1997a; Wilkinson *et al*., 2001a) and between species (Coffey and Bower, 1984; Fenn and Coffey, 1984; Coffey and Joseph, 1985; Ouimette and Coffey, 1989a). It has been suggested that the efficiency of phosphite uptake by fungi may account for some of the observed differences in sensitivity (Barchietto *et al*., 1989).

Phosphate is a competitive inhibitor of phosphite uptake by *Phytophthora* spp. (Barchietto *et al*., 1989; Griffith *et al*., 1989a). As a consequence, the concentration of phosphate present in the growth media influences the fungitoxicity of phosphite, with higher concentrations of phosphite required to inhibit mycelial growth as the

concentration of phosphate increases (Fenn and Coffey, 1984; Griffith *et al*., 1989a; Darakis *et al*., 1997; Komorek and Shearer, 1997). Phosphite also inhibits the fungal uptake of phosphate, although mM concentrations of phosphite are required to inhibit phosphate transport in comparison with the μM concentrations of phosphate that inhibit phosphite transport (Griffith *et al*., 1989b).

Numerous researchers have investigated the site of phosphite action in *Phytophthora* spp., and have found that sub-toxic levels of phosphite result in metabolic changes in the fungi (Dunstan *et al*., 1990; Griffith *et al*., 1990; Niere *et al*., 1990; Barchietto *et al*., 1992; Niere *et al*., 1994). In particular, phosphite alters the cell wall composition of *Phytophthora palmivora* (Dunstan *et al*., 1990) and *P. capsici* (Rouhier *et al*., 1993). Cell wall extracts of fungi exposed to phosphite contain enhanced levels of elicitors (Saindrenan *et al*., 1990; Rouhier *et al*., 1993; Perez *et al*., 1995), which stimulate plant defence responses (Isaac, 1992).

1.7.3 Indirect activity of phosphite

The stimulation of plant defence responses has been observed in several host-pathogen interactions after treatment with phosphite or Fosetyl-Al, with normally susceptible hosts exhibiting responses to pathogens similar to those observed in naturally resistant plants (Guest, 1984; Khan *et al*., 1986; Afek and Sztejnberg, 1989; Guest *et al*., 1989; Nemestothy and Guest, 1990). The cessation of pathogen growth was associated with an increased accumulation of phytoalexins in seedlings of citrus (Khan *et al*., 1986; Afek and Sztejnberg, 1989), cowpea (Saindrenan *et al*., 1988a) and tobacco (Guest, 1984; Nemestothy and Guest, 1990). In *E. marginata*, a decrease in lesion development was associated with increased activity of host defence enzymes and accumulation of soluble phenolics (Jackson *et al*., 2000). Anatomical barriers formed in response to *P. cinnamomi* after treatment with

phosphite in two species usually highly susceptible to the pathogen - *Leucadendron* hybrids (Marks and Smith, 1992) and *Banksia brownii* (Smith *et al*., 1997).

1.7.4 Mode of action

The relative importance of the direct and indirect activity of phosphite was under considerable discussion in the 1980s. To determine the mode of action of phosphite, researchers attempted to compare its *in vitro* and *in vivo* activity. Similarities in the sensitivity of isolates in *in vitro* and *in vivo* tests were considered good evidence to support a direct mode of action (Fenn and Coffey, 1984; Fenn and Coffey, 1985; Dolan and Coffey, 1988; Ouimette and Coffey, 1989b). Levels of phosphite detected in avocado trees were found to be sufficient to account for a direct antifungal effect of phosphite (Ouimette and Coffey, 1989b). However, inhibition of the defence responses of cowpea with the addition of α-aminooxyacetate modified the phosphite-induced resistance response and resulted in complete susceptibility of the host (Saindrenan *et al*, 1988b). In a study of lupins, tobacco and paw-paw, Smillie *et al*. (1989) suggested that although phosphite concentrations were high enough to reduce fungal growth directly, plant defences were likely to contribute to the complete inhibition of the pathogen. Guest (1986) also described the mode of action of Fosetyl-Al as mixed, with the direct and indirect activity allowing a full expression of the genetic potential for resistance in plants. Direct inhibition may be more important in situations where high concentrations of phosphite are present (Afek and Sztejnberg, 1989; Jackson *et al*., 2000), or in plants with poor defence responses (Guest and Grant, 1991).

1.7.5 Detection and distribution of phosphite in plants

Phosphite is translocated in the xylem and phloem of plants (Groussol *et al*., 1986), and is therefore a systemic chemical. Several studies have confirmed the phloem

mobility of phosphite, with the detection of the anion in the roots of various plant species after trunk injection or foliar spraying (Ouimette and Coffey, 1989b; Schutte *et al*., 1991; Whiley *et al*., 1995; Jackson *et al*., 2000). Several methods have been employed to detect phosphite in plant tissue, including radiolabelling (Fenn and Coffey, 1985; d'Arcy-Lameta *et al.*, 1989; Ouimette and Coffey, 1990), gas chromatography (Saindrenan *et al.*, 1985; Smillie *et al.*, 1988; Hargreaves and Ruddle, 1990), and ion chromatography (Ouimette and Coffey, 1988; Smillie *et al.*, 1988; Glenn *et al*., 1990; Roos *et al.*, 1999). A direct comparison of radiolabelling and ion chromatography to detect phosphite in tobacco and tomato found that the results were equivalent for both techniques (Fenn and Coffey, 1989). Although Smillie *et al*. (1988) named gas chromatography as a preferred method over ion chromatography, Roos *et al*. (1999) expressed concerns over the tedious protocols and increased likelihood of chemical loss during sample preparation for gas chromatography. A methodology for High Performance Ion Chromatography (HPIC), described as simple and reliable (Roos *et al*., 1999), was developed to analyse samples for phosphite research at Murdoch University, including the work presented in this thesis.

Research conducted by the Department of Conservation and Land Management (CALM) suggested that phosphite was readily translocated to the roots of native Australian plants, with greater concentrations of phosphite detected in the roots than shoots of *Banksia telmetia* and *Lambertia multiflora* (Komorek and Shearer, 1997). However, phosphite was not detected in the roots of *Xanthorrhoea preissii* and did not have any effect on root colonisation by *P. cinnamomi* (Pilbeam *et al*., 2000). This highlights the need for further work to be done on the distribution of phosphite in native plants after foliar spraying.

1.8 Summary and thesis objectives

Native ecosystems in Australia are under threat from *P. cinnamomi* and there is considerable interest in using phosphite to protect them from the destructive pathogen. A paucity of information on the effect of phosphite on the host-pathogen interaction in Australian plant species has led to uncertainty about the most effective treatment for native plants. In this thesis, *E. marginata* is used as a model plant to examine the effects of phosphite on the host-pathogen interaction. Figure 1.2 illustrates the structure of the thesis and the three key areas that are investigated.

Chapter 2 looks at the sensitivity of *E. marginata* seedlings to different concentrations of phosphite and examines the effect of applied phosphite concentration on the *in vivo* concentration of phosphite in different plant tissues.

The pre-inoculation efficacy of phosphite in protecting *E. marginata* seedlings from stem colonisation by *P. cinnamomi* is then investigated in Chapter 3. In particular, the effects of applied phosphite concentration on the duration of effectiveness are investigated.

Concern over the survival of *P. cinnamomi* in plants treated with phosphite led to an investigation of whether phosphite could prevent the death of infected *E. marginata* seedlings under field conditions (Chapter 4). A dynamic response to infection with *P. cinnamomi* was observed, leading to the determination of resistance mechanisms used by *E. marginata* to protect it from the pathogen.

In a glasshouse trial the effect of phosphite on disease development and anatomical responses of *E. marginata* to wounding and *P. cinnamomi* were examined under controlled conditions (Chapter 5). The effect of temperature on the effectiveness of phosphite against *P. cinnamomi* and anatomical responses of *E. marginata* was also determined (Chapter 6).

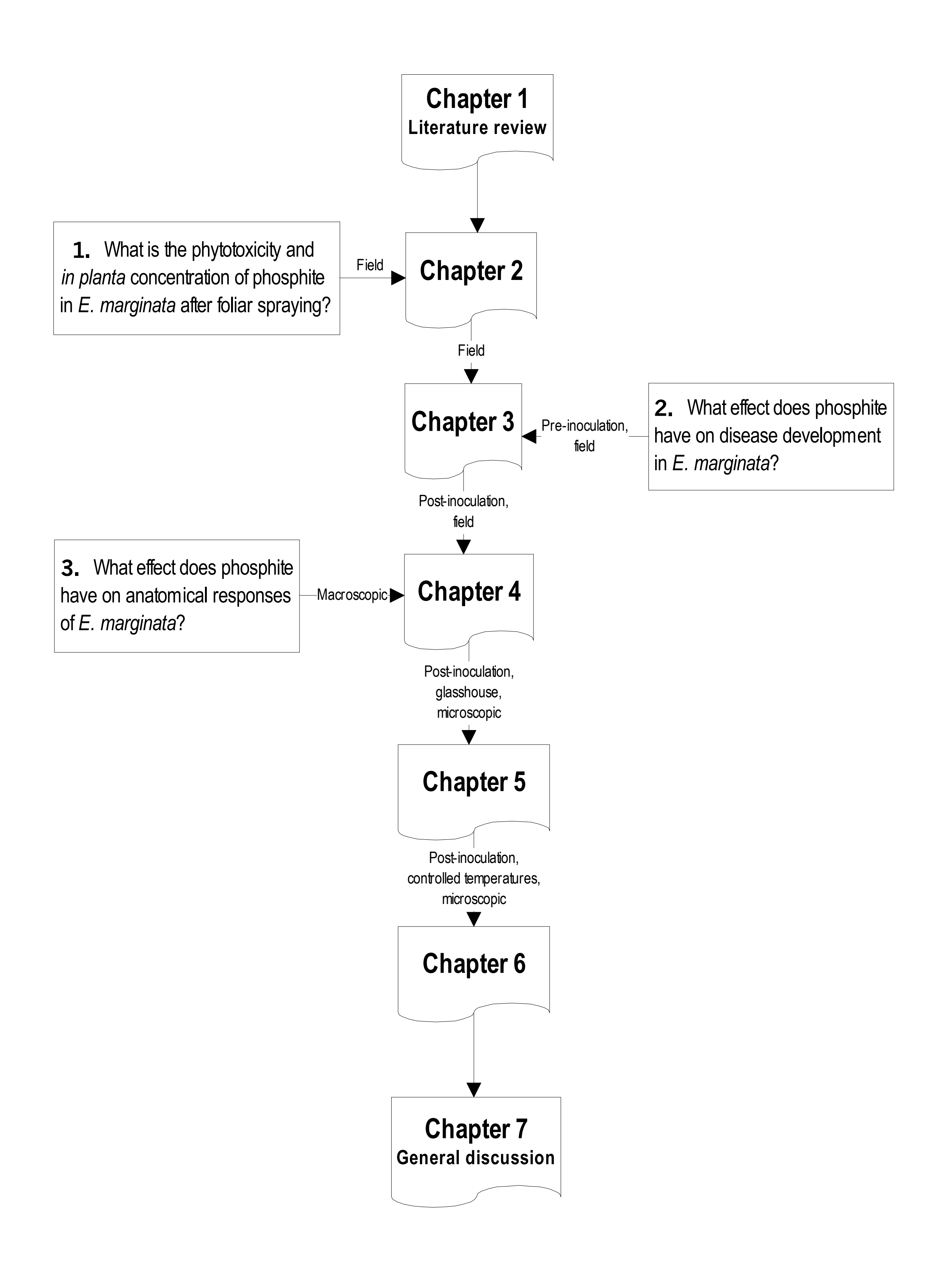


Figure 1.2: Flow diagram of thesis, illustrating the chapters in which the three key elements of this phosphite research feed into the thesis.

Chapter 2: Phosphite phytotoxicity and *in planta* phosphite concentration in *Eucalyptus marginata* seedlings

2.1 Introduction

Although phosphite has been described as having low toxicity to plants (Guest and Grant, 1991), there are a number of reports of the development of phytotoxicity symptoms after the foliar application of phosphite. Leaf burn has developed in mandarin trees (Walker, 1989), almond and cherry trees (Wicks and Hall, 1988; 1990) and carrots (Walker, 1991) in response to spraying with phosphite. Several Australian native plant species have also been found to be sensitive to phosphite (Barrett and Grant, 1998; Aberton *et al*., 1999; Pilbeam *et al*., 2000; Barrett, 2001; Tynan *et al*., 2001).

The phytotoxicity of phosphite to *E. marginata* under field conditions has been examined previously in clonal material (Jackson, 1997b). Severe leaf burn developed in most clonal lines after treatment with 20 g phosphite/L in autumn (up to 80% leaf area was necrotic), while treatment with 5 g phosphite/L led to the development of moderate phytotoxicity symptoms (up to 30% leaf area was damaged) (Jackson, 1997b).

In a previous study, the phytotoxicity of phosphite was found to be positively correlated with the *in planta* concentration of phosphite in the shoots of sprayed *Adenanthos barbiger* and *Daviesia decurrens* (Pilbeam *et al*., 2000). Barrett (2001) detected four times more phosphite in burnt foliage than in unaffected leaves, and suggested that phytotoxicity may hinder the translocation of phosphite to root tissue if substantial quantities of phosphite were locked in burnt areas of leaves.

The aims of the present study were to determine the maximum phosphite concentration which could be applied to the leaves of *E. marginata* in field conditions

without the development of severe phytotoxicity symptoms, and whether applied phosphite concentration influenced the *in planta* phosphite concentration in stems, lignotubers and roots.

2.2 Materials and methods

2.2.1 Experimental design

The independent variables were treatment of *E. marginata* seedlings with 0, 2.5, 5, 7.5 and 10 g phosphite/L (0, 0.25, 0.5, 0.75 and 1% phosphite). The dependent variables were the phytotoxicity ratings at all applied phosphite concentrations, and concentrations of phosphite in various tissue types (lower stem, lignotuber, tap root and lateral root) *in planta* after treatment with 0, 5 and 10 g phosphite/L. The trial had a randomised complete block design, with seven replicate plants sprayed for each phosphite treatment. Six of these replicate plants were selected randomly for phosphite analysis.

2.2.2 Plant material

The trial was conducted in a rehabilitated bauxite minepit located in the Huntly Mine of Alcoa World Alumina Australia, Western Australia (116°6'E and 32°36'S). The mine pit was an active *Phytophthora* site, with *P. cinnamomi* and *P. citricola* known to be present (Gordon Baird, personal communication). The trial was conducted away from the area of recent plant deaths. *E. marginata* had established naturally from seeding of the area approximately 18 months before the trial. Plant height was measured one month prior to phosphite treatment, and ranged from 50-70 cm.

2.2.3 Spray application of phosphite

Plants were sprayed with phosphite in early January 1997 (summer). The maximum temperature on the day of spraying was 28°C. To prevent soil drenching,

newspaper and plastic were placed around the base of each plant. The foliage was then sprayed to run-off with phosphite using a backpack sprayer, which was regularly agitated. The phosphite solutions were prepared from Fosject 200 (Unitec Group Pty Ltd, Australia), a 20% w/v solution of mono-di potassium phosphite. All treatments contained 0.25% Synertrol oil (Organic Crop Protectants Pty Ltd, Australia), which was added to increase spray deposition, droplet spread, penetration and uptake of phosphite.

2.2.4 Phytotoxicity symptoms

Seven days after phosphite treatment, plants were assessed for leaf burn, on a scale of 0-3 (0 = no burning, 1 = 1 to 25% of leaf area burnt, 2 = 26 to 50% of leaf area burnt and 3 = >50% leaf area burnt).

2.2.5 Harvesting plants

Plants treated with 0, 5 and 10 g phosphite/L were harvested 7 days after spraying. The soil was removed from around the base of the plants to expose the upper 15-20 cm of the tap root. The roots were severed at this depth, and each plant was then separated into four tissue types for phosphite analysis – lower stem (approximately 15 cm in length), lignotuber, tap root and lateral roots. Leaf tissue and upper stem were also collected, but could not be analysed due to financial and time constraints.

2.2.6 Phosphite analysis

Plant tissues were washed in warm water with a small amount of phosphate-free detergent (Colgate-Palmolive, Australia) and rinsed in warm water before being dried at 37°C for at least 7 days. They were then ground into a fine powder using an electric grinding machine.

For each replicate, 5 mL water was added to 0.5 g ground plant tissue and left overnight, before the supernatant was removed and filtered through a 0.45 μm Millipore filter. Samples were then analysed for phosphite using HPIC, as described by Roos *et al*. (1999).

2.2.7 Statistical analysis

The correlation between applied phosphite concentration and phytotoxicity rating was determined using the Spearman rank correlation. Using the general linear model procedure, the effects of applied phosphite concentration and tissue type on the *in planta* concentrations of phosphite were analysed by MANOVA. Data residuals were inspected for normality and homogenous variances. The data was then $\log_{10}(x+12)$-transformed prior to analysis (twelve was the lowest concentration of phosphite detected in the samples). The interaction between applied phosphite concentration and tissue type was also tested. Pairwise comparisons were made using the Tukey HSD test.

2.3 Results

2.3.1 Phytotoxicity

Severe leaf burn developed in *E. marginata* within 7 days of foliar treatment with 7.5 and 10 g phosphite/L (Table 2.1), with damage to 64 and 75% leaf area, respectively. Plants treated with 2.5 and 5 g phosphite/L had damage to 16 and 43% leaf area, respectively. The correlation of phytotoxicity rating with applied phosphite concentration was strongly positive, with $r_s = +0.94$ ($P<0.001$). Plants treated with zero phosphite did not develop any phytotoxicity symptoms.

2.3.2 *In planta* phosphite concentrations

Overall, phosphite concentrations detected in plants treated with 10 g phosphite/L were not significantly (P=0.07) higher than in the 5 g phosphite/L treatment, despite a two-fold difference in the concentration of phosphite in stems (Figure 2.1). The concentration of phosphite detected was significantly (P<0.001) different among tissue types, with significantly greater concentrations of phosphite in the stems of *E. marginata* than in the lignotubers and roots (Figure 2.1). There was no significant (P=0.4) interaction in the effect of applied phosphite concentration and tissue type on *in planta* phosphite concentration. The range of average phosphite concentrations *in planta* was from 27.6 μg/g dry weight (in the tap roots, 5 g phosphite/L treatment) to 423.4 μg/g dry weight (in the stem, 10 g phosphite/L treatment). No phosphite was detected in plants treated with zero phosphite.

Table 2.1: Phytotoxicity ratings in 18-month old *Eucalyptus marginata* seedlings 7 days after the foliar application of phosphite.

Ratings: 0 = no burning, 1 = 1 to 25% of leaf area burnt, 2 = 26 to 50% of leaf area burnt and 3 = >50% leaf area burnt.

Applied phosphite concentration (g/L)	Average phytotoxicity rating (n=7)
0	0
2.5	1.1
5	2.1
7.5	2.7
10	3.0



Figure 2.1: Mean phosphite concentrations (µg/g dry weight) detected in various tissue types of *Eucalyptus marginata* seedlings 7 days after foliar treatment with 5 (□) or 10 (▤) g phosphite/L. Bars represent the standard errors of the means (n=6).

2.4 Discussion

The detection of phosphite in the stems and roots of *E. marginata* within 7 days of spraying showed that phosphite was readily translocated to plant parts which may be under threat from *P. cinnamomi*, particularly the lower stem, which is reported to be the site of initial infection of *E. marginata* in rehabilitated sites (Hardy *et al*., 1996). The detection of a higher concentration of phosphite in the stems than in the roots of *E. marginata* is similar to the findings of Ouimette and Coffey (1989b) following the foliar treatment of *Persea americana* seedlings with 2.1 g phosphite/L. In *P. americana*, the concentration of phosphite detected in the roots was consistently less than in the stems and leaves up to 8 weeks after treatment (Ouimette and Coffey, 1989b). In contrast, Komorek and Shearer (1997) reported a significantly higher concentration of phosphite in the roots than in the shoots of *Banksia telmetia* and *Lambertia multiflora* within one year of spraying. The relative distribution of phosphite in various plant tissue types has been found to vary temporally (Schutte *et al*., 1991) and seasonally (Whiley *et al*., 1995). In citrus trees, greater concentrations of phosphite were detected in the leaves until 28 days after spraying, at which time the roots contained more phosphite than the leaves (Schutte *et al*., 1991). After tree injection of *P. americana*, the length of time before roots contained more phosphite than leaves was dependent upon the season of injection, with more efficient translocation of phosphite to the roots when shoots were not a strong sink for photoassimilates (Whiley *et al*., 1995). The sink/source status of shoots and roots in *E. marginata* during the present study is not known. Stoneman (1992) noted that the root:shoot ratio in *E. marginata* growing in rehabilitated mine pits in January was highly dependent upon the soil temperature and moisture, neither of which was measured in the current study.

Analysis of *E. marginata* at 7 days after phosphite treatment provided a limited picture of the distribution of phosphite. It showed, however, that the applied

phosphite concentration did not influence the *in planta* phosphite concentration in the tissues analysed. In particular, the levels detected in the roots were very similar in the two treatments. This differs from the results of Fairbanks *et al*. (2000), in which *Corymbia calophylla* seedlings were treated with 0, 2.5, 5 and 10 g phosphite/L and harvested after 7 days. Every tissue analysed (roots, stems and leaves) had an increase in the amount of phosphite detected with an increase in the applied phosphite concentration (Fairbanks *et al*., 2000). Komorek and Shearer (1997) also found levels of phosphite to increase in roots with greater concentrations of applied phosphite. It seems unlikely that the leaf phytotoxicity of treatment with 10 g phosphite/L hindered the distribution of phosphite to the roots in the present study, since there was double the concentration of phosphite in the stems of plants treated with 10 g phosphite/L than in the 5 g phosphite/L treatment. It is unfortunate that the leaf tissue could not be analysed, as this would have given a more complete picture of the short-term distribution of phosphite in *E. marginata*, and may have provided a different view of the effect of applied phosphite concentration on the *in planta* phosphite concentration.

Although the lignotubers of eucalypts have been described as food storage tissues (Bamber and Mullette, 1978) and carbohydrate partitioning influences the distribution of phosphite (Whiley *et al*, 1995), the lignotubers of *E. marginata* were not a major storage area for phosphite at 7 days after treatment.

The HPIC system has been reported to be a convenient and inexpensive method for the analysis of phosphite concentrations (Roos *et al*., 1999). It was mentioned, however, that the method could only be accurate for concentrations of phosphite above 3-5 ppm (Roos *et al*., 1999). In the present study, 42% of tap root samples contained less than 3 ppm phosphite (equivalent to 30 μg phosphite/g dry weight). The mean phosphite concentration presented for the tap roots is therefore

likely to be imprecise, but at least provides a rough estimate of the phosphite concentration in the tap roots relative to the other tissues analysed.

The highest concentration of phosphite applied to *E. marginata* that did not result in the development of severe leaf phytotoxicity symptoms was 5 g phosphite/L. As supported by the findings of Jackson (1997b), treatment with 5 g phosphite/L was moderately phytotoxic to *E. marginata*. Very little phytotoxicity was observed after treatment with 2.5 g phosphite/L. Having selected 2.5 and 5 g phosphite/L as appropriate concentrations for treating *E. marginata*, the next chapter will investigate whether foliar treatment with 2.5 or 5 g phosphite/L is effective against the colonisation of *E. marginata* stems by *P. cinnamomi*.

Chapter 3: The extension of *Phytophthora cinnamomi* in *Eucalyptus marginata* stems inoculated at different times after treatment with phosphite

3.1 Introduction

The duration of the effect of phosphite has mainly been examined in horticultural plant species. In cherry trees inoculated with *P. cambivora*, phosphite was reported to inhibit lesion development for at least 17 weeks after the foliar application of 1 g phosphite/L or trunk injection of 100 g phosphite/L (Wicks and Hall, 1988). In apple trees infected with *P. cactorum*, injection with 4 g a.i. of fosetyl-Al restricted lesion development for at least 15 months after application (Long *et al.*, 1989). The recommended application interval of phosphite for the optimum control of *Phytophthora* diseases in cocoa is 6-monthly for the first year, then annual injections thereafter unless there is a high disease pressure, where 6-monthly intervals may be necessary (Guest *et al.*, 1994). Phosphite has been found to control root rot caused by *P. cinnamomi* in avocados for 12 months after injection with 200 g phosphite/L (Pegg *et al.*, 1985), though injecting with phosphite twice a year is recommended under high disease pressure (Whiley *et al.*, 1988).

Shearer and Fairman (1997a) suggested that phosphite may have a much longer period of effectiveness against *P. cinnamomi* in natural plant communities in south-western Australia than in horticultural plants. Phosphite was shown to protect *Banksia grandis* and *E. marginata* trees from *P. cinnamomi* lesion extension for at least four years after trunk injections with 50, 100 and 200 g phosphite/L, with little initial difference in efficacy between phosphite concentrations (Shearer and Fairman, 1997a). By 6 years after phosphite injection, there was an apparent response to applied phosphite concentration, with the highest treatment resulting in

the least lesion extension. In another study by Shearer and Fairman (1997b), treatment with 2.5 or 5 g phosphite/L sprayed to run-off reduced the mortality of three *Banksia* species. However, there was 50% mortality of *B. brownii* within 3 years in both treatments (Shearer and Fairman, 1997b). Komorek and Shearer (1997) found that *B. menziesii* and *B. attenuata* plants growing on the edge of an active *P. cinnamomi* front and treated with 200 g phosphite/L twice or 400 g phosphite/L once or twice using an ultra-low volume sprayer remained healthy for at least 2 years. Plant mortalities were observed within one year of treatment with 100 g phosphite/L sprayed twice or 200 g phosphite/L sprayed once (Komorek and Shearer, 1997).

The present study was conducted to determine the optimum applied phosphite concentration and the duration of phosphite effectiveness in protecting young growing *E. marginata* plants from colonisation by *P. cinnamomi* after new challenges with the pathogen up to 17 months after the foliar application of phosphite.

3.2 Materials and methods

3.2.1 Experimental design

The independent variables were treatment of *E. marginata* seedlings with 0, 2.5 and 5 g phosphite/L (0, 0.25 and 0.5% phosphite), and time of inoculation with *P. cinnamomi* after spraying (6 days and 6, 12 and 17 months). The dependent variable was the extent of colonisation by *P. cinnamomi* above the inoculation point at 19-21 days after each inoculation. The trial had a randomised complete block design. At each inoculation time, there were nine replicate plants inoculated with *P. cinnamomi* for each phosphite concentration, plus nine control sham-inoculations.

For phosphite analysis, the independent variables were the time after spraying the foliage with 5 g phosphite/L (6 days and 6 months, plus 19-21 days) and tissue type (leaf, stem and tap root). The dependent variable was the *in planta*

concentration of phosphite. Three and six replicate plants were selected randomly from nine plants inoculated at 6 days and 6 months after spraying, respectively. Three and six replicate plants receiving the 0 g phosphite/L treatment served as a negative control.

3.2.2 Plant material and growth

The trial was conducted in a rehabilitated bauxite mine pit (Alcoa World Alumina Australia, Jarrahdale Mine, Western Australia; 116°7'E and 32°22'S). The plants had established naturally from seeding of the area after mining. At the commencement of the trial, the *E. marginata* seedlings were approximately one-year-old, with plant heights ranging from 30-75 cm and stem diameters ranging from 0.5-2 cm. Since *P. cinnamomi* was present at the site, plants growing at the top of rip lines were selected to minimise the chance of natural infection of the collar.

Plant height and stem diameter at 5 cm above soil level were measured at 6-monthly intervals, just prior to inoculation with *P. cinnamomi*.

3.2.3 Spray application of phosphite

Plants were sprayed in late October 1997 (spring). Prior to spraying, the soil surrounding each plant was covered with absorbent paper and woollen material to prevent soil drenching. The foliage was then sprayed to run-off with phosphite using a backpack sprayer. The phosphite solutions were prepared from Fosject 200 (Unitec Group Pty Ltd, Australia), a 20% w/v solution of mono-di potassium phosphite. All treatments contained 0.25% Synertrol oil (Organic Crop Protectants Pty Ltd, Australia), which was added to increase spray deposition, droplet spread, penetration and uptake of phosphite.

3.2.4 Phytotoxicity symptoms

Six days after spraying, the plants were assessed for leaf burn, on a scale of 0-3 (0 = no burning, 1 = 1 to 25% of leaf area burnt, 2 = 25 to 50% of leaf area burnt and 3 = >50% leaf area burnt).

3.2.5 Inoculum material and inoculation procedure

P. cinnamomi isolate MP94-48 (Murdoch Culture Collection) was used. This isolate has previously been found to form large lesions in excised stems of *E. marginata* (Hüberli, 1995), and is moderately sensitive to phosphite *in vitro* (Wilkinson *et al.*, 2001a). The isolate was passaged through *E. marginata* one month before inoculation to ensure it had not lost its pathogenicity in culture and then maintained on half strength potato dextrose agar (½PDA) prior to use. Colonised Miracloth (Calbiochem, USA) discs were prepared by placing agar plugs colonised with *P. cinnamomi* around sterile 5 mm diameter Miracloth discs on ½PDA plates. Plates were incubated at 23°C in the dark. Colonised discs were used within 7 days, and sterile Miracloth discs were used in the control inoculations.

At 6 days (spring) and 6 (autumn), 12 (spring) and 17 months (autumn) after spraying, stems of 36 *E. marginata* plants were inoculated 10 cm above the soil level. Using a sterile razor blade, a cut (approximately 6 mm long and 3 mm wide) was made through the bark tissue in an upward direction, leaving the flap attached to the stem at the upper edge of the cut. A Miracloth disc was placed inside each opening and the inoculation point was then sealed with Parafilm (American National Can, USA) and wrapped with plastic flagging tape.

3.2.6 Harvesting plants

Plants were harvested 19-21 days after each inoculation. This time was selected after the first inoculation to allow substantial lesion development but not give the

pathogen enough time to reach the stem apex. Lesion lengths above and below the inoculation point were measured separately. Recovered Miracloth discs and stems were plated onto NARPH, a *Phytophthora*-selective agar (Hüberli *et al.*, 2000). Where lesions were visible, two 1 cm segments into the lesion and 1 cm segments up to 8 cm beyond the lesion were cut in half longitudinally and plated sequentially. In the absence of a visible lesion, 1 cm segments were cut up to 10 cm beyond the inoculation point. In harvest one, the sectioning started at the middle of the inoculation point, while in subsequent harvests, a 1 cm segment which incorporated the entire inoculation point was cut and plated separately from the ten 1 cm segments. This change in the method made it easier to identify plants in which *P. cinnamomi* colonisation was restricted to the inoculation point.

Plates were incubated at 23°C for 8 days and examined every second day for the growth of *P. cinnamomi*. Segments from which *P. cinnamomi* was isolated were removed (including the surrounding agar) to prevent contamination of other segments on the plate.

The extent of colonisation of stems by *P. cinnamomi* was calculated by adding the total lesion length to the length of stem segments beyond the lesion from which *P. cinnamomi* was isolated. The extent of colonisation above the inoculation point was calculated separately from that below the inoculation point.

3.2.7 Phosphite analysis

Approximately 10-20 g of leaves and tap roots were sampled for phosphite analysis, plus the portion of stem remaining after segments were plated for *P. cinnamomi*. Samples were prepared and analysed as described in Section 2.2.6.

3.2.8 Environmental conditions and water relations

Daily maximum and minimum temperatures for the time periods between inoculation

and harvest of plants were obtained from the Bureau of Meteorology, Karnet Station No. 009111. Daily rainfall data was provided by Alcoa World Alumina Australia from the Jarrahdale Mine.

The water relations of nine non-inoculated plants treated with 0 g phosphite/L were measured at each inoculation time. Pre-dawn and midday xylem pressure potentials were measured using the pressure chamber method of Scholander *et al.* (1965). The youngest fully expanded leaf was cut from each plant using a sharp razor blade, then immediately wrapped in foil and stored in an airtight container before the leaves from each of the nine plants were transported to the pressure chamber. Measurements were made within ten minutes of leaf collection.

3.2.9 Statistical analysis

An analysis of variance was conducted to compare the xylem pressure potentials at different inoculation times, and to test the effect of phosphite treatment on shoot height or stem diameter.

Stem colonisation data were $\log_{10}(x+1)$ transformed to make residuals homoscedastic and approximately normal. The data were then analysed by ANCOVA (with stem diameter as a covariate) to determine if there was any significant difference between *P. cinnamomi* extension up or down from the inoculation point. Since there was no significant difference ($P>0.05$) between *P. cinnamomi* extension in the two directions, further statistical tests were conducted on the extent of colonisation by *P. cinnamomi* above the inoculation point only. Plants from which *P. cinnamomi* was not isolated were removed from the dataset (including four plants from each phosphite treatment at 17 months). Using the general linear model procedure, the log-transformed colonisation data was analysed by MANCOVA to determine the effect of phosphite concentration and time of inoculation on the extent of stem colonisation by *P. cinnamomi*. Stem diameter was

used as a covariate and the block effect was included in the calculation of the error term. Least Squares Means were used (P=0.05) to determine the effect of phosphite concentration on the extent of colonisation at each inoculation time.

Using the general linear model procedure, the effects of time after spraying and tissue type on *in planta* phosphite concentration were analysed by MANCOVA, with stem diameter as a covariate. The data was $\log_{10}(x+12)$-transformed to satisfy the assumptions of normality.

3.3 Results

3.3.1 Environmental conditions and water relations

Average ambient temperatures recorded at Karnet between inoculation and harvest times were 17, 16, 19 and 21°C, with temperatures ranging from 7-30, 6-28, 6-35 and 10-32°C, at 6 days and 6, 12 and 17 months after phosphite treatment, respectively (Figure 3.1).

Rainfall during the 2 months previous to inoculation at 6 days and 6, 12 and 17 months after phosphite treatment totalled 174.4, 91.8, 265.2 and 28.6 mm, respectively. Between inoculation and harvest times, plants inoculated 12 months after treatment received the largest amount of rainfall (49 mm), while the lowest rainfall (2.8 mm) occurred at 17 months after treatment (Figure 3.1).

The pre-dawn water relations of the plants inoculated in autumn (6 and 17 months after spraying) were lower than those inoculated in spring (6 days and 12 months after spraying) (Table 3.1). The plants inoculated at 17 months after spraying had the lowest xylem pressure potential, with pre-dawn and midday water potentials of –1.3 MPa and –2.6 MPa, respectively.

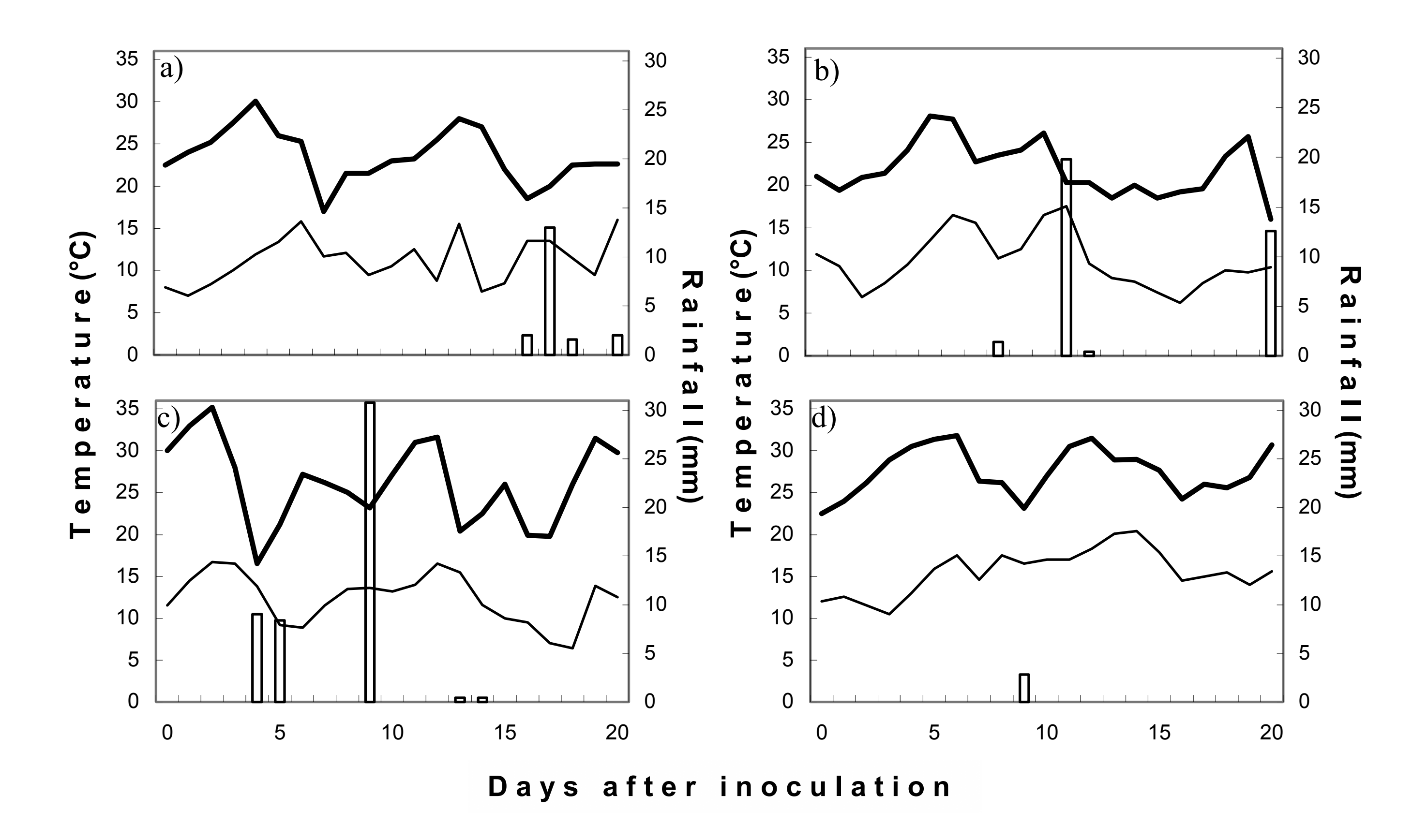


Figure 3.1: Daily maximum (▬) and minimum (—) temperatures at Karnet and daily rainfall (□) at Jarrahdale after inoculation of *Eucalyptus marginata* with *Phytophthora cinnamomi* and until plants were harvested. Time of inoculation: a) 6 days, b) 6 months, c) 12 months and d) 17 months after phosphite treatment.

Table 3.1: Mean pre-dawn and midday xylem pressure potentials (XPP) of *Eucalyptus marginata* treated with 0 g phosphite/L just prior to each time of inoculation (± standard error of the mean). Means followed by the same letter within columns are not significantly different (P>0.05).

Time after phosphite treatment	Season	Pre-dawn XPP (MPa)	Midday XPP (MPa)
6 days	spring	- 0.4 ± 0.1a	- 1.8 ± 0.1a
6 months	autumn	- 0.9 ± 0.2ab	- 1.7 ± 0.1a
12 months	spring	- 0.5 ± 0.1a	- 1.5 ± 0.1a
17 months	autumn	- 1.3 ± 0.2b	- 2.6 ± 0.2b

3.3.2 Plant growth

There was no significant (P>0.05) difference in shoot height or stem diameter between plants treated with 0, 2.5 or 5 g phosphite/L at any of the inoculation times. Shoot height of plants inoculated 17 months after phosphite treatment (data combined for all treatments) was treble that of plants inoculated 6 days after treatment, while stem diameter doubled over the 17 months (Figure 3.2).

3.3.3 Phytotoxicity symptoms

Some leaf burning was evident in *E. marginata* plants treated with 2.5 and 5 g phosphite/L. The average phytotoxicity ratings were 0, 0.5 and 1.3 for treatments 0, 2.5 and 5 g phosphite/L, respectively. Only two plants had greater than 50% damage to the leaf area, and these were both treated with 5 g phosphite/L. Overall, the phytotoxicity symptoms were minimal.

3.3.4 Lesion lengths in stems of *E. marginata*

Black lesions formed on the stems of untreated *E. marginata* after the first inoculation with *P. cinnamomi*, with a mean lesion extension of 77±17 mm above the inoculation point. No lesions were evident on the plants treated with 2.5 or 5 g phosphite/L, or in the plants that were sham-inoculated.

In the subsequent inoculations, a total of three plants developed a lesion, with one in each phosphite treatment.

3.3.5 Colonisation of *E. marginata* stems by *P. cinnamomi*

The applied phosphite concentration and time of inoculation after phosphite treatment each had a significant (P<0.001) effect on the extent of colonisation by

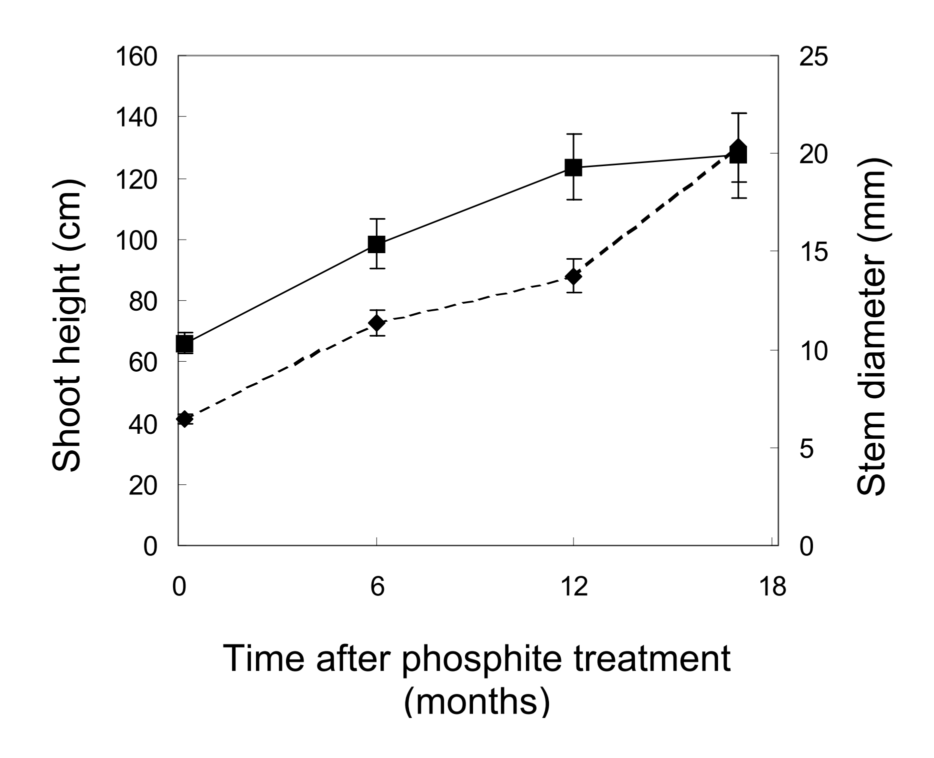


Figure 3.2: Shoot height (- -◆- -) and stem diameter (—■—) of *Eucalyptus marginata* plants at the time of each inoculation. Values are means of 27 plants (± standard error of the mean), with all phosphite treatments combined.

P. cinnamomi. The least colonisation occurred in all treatments after the second inoculation (Figure 3.3). *P. cinnamomi* was not isolated from any of the plants inoculated with sterile Miracloth discs.

3.3.5.1 6 days after phosphite treatment

Colonisation by *P. cinnamomi* in plants inoculated 6 days after treatment with 2.5 or 5 g phosphite/L was significantly less than that observed in plants treated with 0 g phosphite/L (Figure 3.3). There was no significant difference in the extent of colonisation between the 2.5 and 5 g phosphite/L treatments. In comparison with the highly variable colonisation in plants receiving the 0 g phosphite/L treatment, there was very little variability in the 2.5 or 5 g phosphite/L treatments. In the untreated plants, colonisation by *P. cinnamomi* extended up to 30 mm beyond the visible lesion. *P. cinnamomi* was isolated further than 10 mm beyond the inoculation point in just one plant treated with 2.5 g phosphite/L, and was never isolated further than 10 mm beyond the inoculation point in the 5 g phosphite/L treatment. *P. cinnamomi* was isolated from all plants (with the exception of one plant treated with 2.5 g phosphite/L).

3.3.5.2 6 months after phosphite treatment

In comparison with the control treatment, the 2.5 and 5 g phosphite/L treatments had a significant effect on the extent of stem colonisation after inoculation with *P. cinnamomi* at 6 months after phosphite treatment. At this time, plants treated with 5 g phosphite/L had significantly less colonisation than those sprayed with 2.5 g phosphite/L (Figure 3.3). *P. cinnamomi* was isolated from all plants (with the exception of one plant treated with 5 g phosphite/L) but was never isolated from beyond the inoculation point in the 5 g phosphite/L treatment.

3.3.5.3 12 months after phosphite treatment

By 12 months after phosphite treatment, colonisation in plants treated with 2.5 g phosphite/L was not significantly less than in those sprayed with 0 g phosphite/L (Figure 3.3). Colonisation was significantly reduced in plants sprayed with 5 g phosphite/L in comparison with the 0 g phosphite treatment. *P. cinnamomi* was isolated from 89% of the plants, with three plants not yielding *P. cinnamomi* (two receiving 0 g phosphite/L treatment and one treated with 5 g phosphite/L).

3.3.5.4 17 months after phosphite treatment

There was no significant treatment effect on stem colonisation by *P. cinnamomi* after inoculation 17 months after phosphite treatment (Figure 3.3). The extent of stem colonisation by *P. cinnamomi* was highly variable in all treatments. The inoculation at this time was not particularly successful, with an isolation of *P. cinnamomi* from only 55.6% of plants in each treatment. This corresponded with a 70-80% recovery of *P. cinnamomi* from the Miracloth discs, in comparison with the 100% recovery obtained after the other inoculations.

3.3.6 *In planta* phosphite concentrations

Neither tissue type nor time after spraying plants with 5 g phosphite/L had a significant (P=0.9) effect on the *in planta* concentration of phosphite detected in *E. marginata*. In the plants sampled within 1 month of spraying, the greatest concentration of phosphite (104 µg/L dry weight) was detected in the leaves (Figure 3.4). By 6 months after spraying, tap roots contained the largest concentration of phosphite (115 µg/L dry weight).

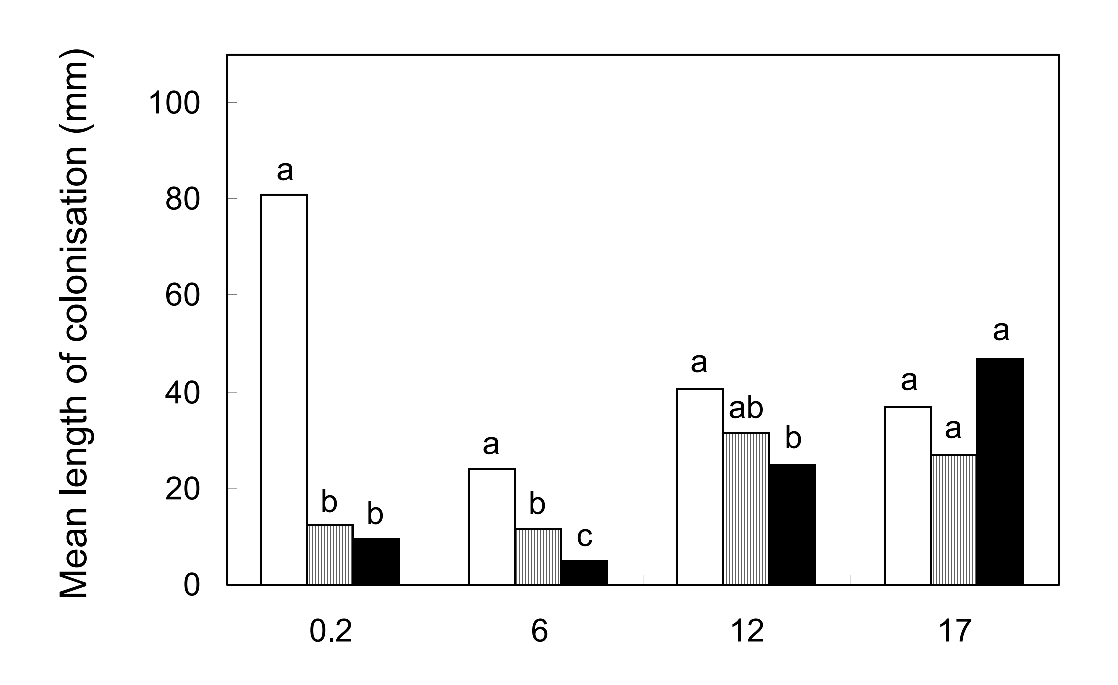


Figure 3.3: Effect of time of inoculation after phosphite treatment and applied phosphite concentration [0 (□), 2.5 (▥) and 5 g phosphite/L (■)] on mean length of colonisation by *Phytophthora cinnamomi* in stems of *Eucalyptus marginata*. Means with different letters indicate a significant ($P<0.05$) difference within each time of inoculation (n=9 for 0.2, 6 and 12 months and n=5 for 17 months).

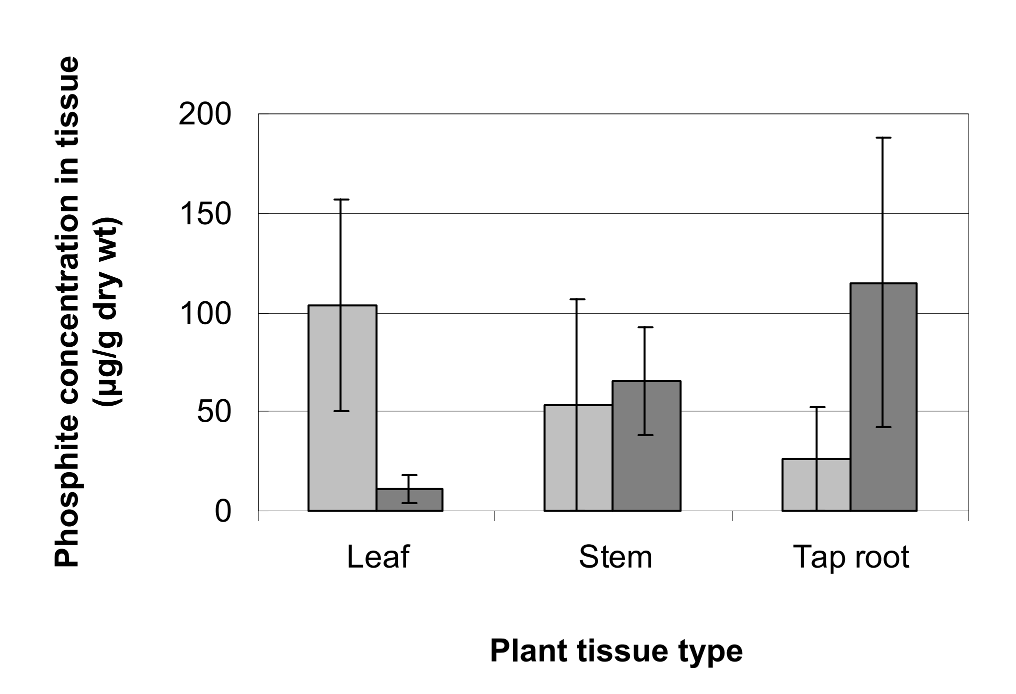


Figure 3.4: Mean phosphite concentration (µg/g dry weight) detected in leaves, stems and tap roots of *Eucalyptus marginata* seedlings inoculated with *P. cinnamomi* at 6 days (□) and 6 months (■) after foliar treatment with 5 g phosphite/L. Plants were harvested 19-21 days after each inoculation. Bars represent the standard errors of the means (n=3 for 6 days and n=6 for 6 months).

3.4 Discussion

Phosphite was initially effective at restricting *P. cinnamomi* extension in stems of *E. marginata* seedlings, but the duration of effect was much shorter than that observed by Shearer and Fairman (1997a) following trunk injection of *E. marginata* trees. Plant age and vigour may have contributed to the observed differences in the duration of efficacy. The young plants used in the present study were actively growing, with a trebling of their height in 17 months. Analysis of *Banksia telmetia* seedlings showed that phosphite concentration decreased considerably within 6 months of treatment, most likely due to the dilution of phosphite as a consequence of a high growth rate (Komorek and Shearer, 1997). It is unfortunate that phosphite analysis of tissue in the present study was limited to one treatment and the two harvest times when phosphite was still effective. However, it is likely that the highest phosphite treatment led to a larger concentration of phosphite in the stems initially, since other studies have shown that treatment with greater concentrations of phosphite leads to a higher concentration of phosphite residues in the stem (Section 2.3.2, Chapter Two; Fairbanks *et al*., 2000). At 6 days and 6 months after spraying, the 2.5 and 5 g phosphite/L treatments were both sufficient to inhibit extension by *P. cinnamomi*. However, after 12 months of active plant growth, it is probable that phosphite residues had declined in plants treated with 2.5 g phosphite/L to a concentration below the effective threshold.

Studies in cocoa trees have shown that the method of application can influence the effectiveness of phosphite. Trunk injections of cocoa trees with 8 or 16 g phosphite/tree were found to be effective against *Phytophthora* pod rot while foliar sprays with 16 or 20 g phosphite/tree did not significantly affect the incidence of pod rot (Anderson and Guest, 1990; Holderness, 1990). It is possible that trunk injection leads to a greater uptake and distribution of phosphite than foliar spraying in some plant species, which could further explain the observed differences in the

duration of effect of phosphite in *E. marginata* between the present study and the results of Shearer and Fairman (1997a).

The spray treatments used by Komorek and Shearer (1997) on *Banksia menziesii* and *B. attenuata* are more comparable with those in the present study. Breakdown in phosphite efficacy against *P. cinnamomi* occurred 12 months after the foliar application of 100 g phosphite/L twice or 200 g phosphite/L once (Komorek and Shearer, 1997), which is the time at which treatment with 2.5 g phosphite/L was no longer effective in restricting the extension of *P. cinnamomi* in *E. marginata* in the present study. According to Fairbanks *et al*. (2000), the ultra-low volume sprays of 100 and 200 g phosphite/L could be equated approximately with sprays to run-off with 5 and 10 g phosphite/L, respectively, when phosphite concentrations in shoot tips were compared in *Corymbia calophylla*.

Although phosphite restricted the extension of *P. cinnamomi* in *E. marginata* inoculated 6 days after treatment, it did not prevent the establishment and survival of *P. cinnamomi* in the host tissue during the three weeks after inoculation. This result supports the findings of Shearer and Tippett (1989), Marks and Smith (1992), Smith (1994) and Pilbeam *et al*. (2000), in which phosphite was effective at reducing lesion lengths but did not kill *P. cinnamomi*. However, the maximum time period between inoculation and harvest in these papers was six weeks. The long-term viability of *P. cinnamomi* in phosphite-treated plants is not known, and is investigated in Chapter 4.

Although *P. cinnamomi* is generally considered to be a necrotroph (Guest and Brown, 1997b), the isolation of *P. cinnamomi* in advance of the lesion and in the absence of a visible lesion suggests that *P. cinnamomi* may be a hemi-biotroph, initially growing biotrophically before becoming necrotrophic (Guest and Brown, 1997b). There have been a number of recent reports of the hemi-biotrophic growth of *P. cinnamomi*, including work on *Pinus radiata* (Davison *et al.*, 1994) and

E. marginata (Davison *et al.*, 1994; O'Gara *et al.*, 1997 and Hüberli *et al.*, 2000). This highlights the importance of isolating the pathogen to estimate colonisation by *P. cinnamomi*, rather than relying solely on the measurement of lesion lengths. It is not known what factors contributed to the apparent differences in the relationship between *P. cinnamomi* and *E. marginata* after inoculation at various times in the present study. There were no obvious differences in the rainfall or temperatures recorded between the first inoculation time (predominantly necrotrophic growth) and the other inoculations (mostly biotrophic).

In the present study, there were large differences in the extent of colonisation in the control plants (treated with zero phosphite) between inoculation times. Previous studies have shown that the season of inoculation can influence the extent of lesion development by *P. cinnamomi* (Tippett *et al*., 1985; Shearer *et al.*, 1988; Davison *et al*., 1994; and Robin *et al.*, 1994). The restricted colonisation after inoculation 6 months after phosphite treatment (autumn) suggests that some factor other than phosphite was influencing colonisation by *P. cinnamomi* at this time. Although the temperature was marginally lower at 6 months after treatment, temperature alone does not explain the observed differences in the extent of colonisation in the control plants. At 17 months after phosphite treatment, it is likely that water stress contributed to the lower isolation of *P. cinnamomi* from *E. marginata*. The water potential measured just prior to inoculation was 0.7 MPa lower than the water potential at which Tippett *et al*. (1987) reported a cessation of lesion development in *E. marginata* (indicative of even greater water stress). The warm conditions and minimal rainfall between the time of inoculation and harvest would most likely have increased the water stress.

The observed longer duration of effect of phosphite at the highest phosphite concentration in the present study and in other studies (Komorek and Shearer, 1997; Shearer and Fairman, 1997a) shows that for the long-term protection of plants

from colonisation by *P. cinnamomi*, it is beneficial to use the highest concentration possible, though severe phytotoxicity should be avoided. Due to the development of severe phytotoxicity symptoms in *E. marginata* after treatment with 7.5 g phosphite/L (Section 2.3.1), treatment using phosphite concentrations greater than 5 g phosphite/L is not recommended if *E. marginata* is sprayed to run-off. It would appear that phosphite needs to be re-applied every twelve months for the continued protection of *E. marginata* from colonisation by *P. cinnamomi*.

Chapter 4: Post-inoculation efficacy of phosphite against *Phytophthora cinnamomi* in stems of *Eucalyptus marginata* seedlings under field conditions

4.1 Introduction

Phosphite has the potential for both preventative and curative treatments for root diseases (Guest and Grant, 1991). In Chapter 3, the prophylactic nature of phosphite was shown by the restricted the extension of *P. cinnamomi* in the stems of *E. marginata* seedlings treated with phosphite. The pathogen did, however, remain viable during the short time between inoculation and harvest.

Field studies of the curative properties of phosphite have focussed on how effectively the chemical can reduce disease development when it is applied post-inoculation. The post-inoculation efficacy of phosphite was found to depend on the severity of disease at the time of treatment in the clover-*P. clandestina* (Greenhalgh *et al*., 1994) and *Xanthorrhoea australis-P. cinnamomi* (Aberton *et al*., 1999) interactions. Phosphite treatment was ineffective if applied when plants exhibited severe disease symptoms. In contrast, avocado trees in an advanced state of decline from root rot caused by *P. cinnamomi* attained a 100% improvement in health by 15 months after phosphite treatment (Pegg *et al*., 1985). Linear extension of lesions was reduced in peach trees following the application of phosphite a week after inoculation with *P. cactorum* (Lim *et al*., 1990).

The current study aimed to determine a) whether phosphite has curative properties in the *E. marginata-P. cinnamomi* interaction and b) the macroscopic responses of *E. marginata* inoculated with *P. cinnamomi*. To determine these, the effects of post-inoculation phosphite treatment on lesion extension, plant survival and pathogen viability were examined.

4.2 Materials and methods

4.2.1 Experimental design

Two trials were conducted to examine the post-inoculation efficacy of phosphite. In the first trial, the independent variable was treatment of *E. marginata* seedlings with 0, 2.5 and 5 g phosphite/L 14 days after inoculation with *P. cinnamomi*. The dependent variables were the total lesion length, lesion extension after phosphite treatment, plant mortality and pathogen viability. The trial had a randomised complete block design. There were 24 replicate plants for each treatment, plus 24 uninoculated plants not treated with phosphite.

The second trial was conducted 7 weeks later with a similar design, except that phosphite treatment was applied 8 days after inoculation, and lesion extension was not a dependent variable due to a difficulty in measuring lesions accurately.

4.2.2 Plant material

The trials were conducted in a rehabilitated bauxite minepit (Alcoa World Alumina Australia, Jarrahdale Mine, Western Australia; 116°7'E,32°22'S). At the commencement of the trials, the *E. marginata* seedlings were approximately one-year-old, with plant heights ranging from 41 - 112 cm and stem diameters ranging from 0.8 – 2.9 cm at 10 cm above the soil level.

4.2.3 Inoculum material and inoculation procedure

The inoculum material was the same as that described in Section 3.2.5, with Miracloth discs colonised with *P. cinnamomi* isolate MP94-48. Sterile Miracloth discs were used in the uninoculated plants.

Plants were inoculated in early December 1998 and late January 1999 (summer) in the first and second trials, respectively. The same method was used as described in Section 3.2.5. Extra plants (18) were inoculated in each trial to assess

the establishment of *P. cinnamomi* prior to phosphite treatment.

4.2.4 Spray application

Plants were sprayed with phosphite 14 and 8 days after inoculation in the first and second trials, respectively. The soil surrounding each plant was covered with a woollen blanket to prevent soil drenching. The foliage was then sprayed to run-off with phosphite using a backpack sprayer. See Section 2.2.3 for details of spray contents.

4.2.5 Monitoring

In the first trial, lesion length above the inoculation point and the extent of girdling were recorded on the day of spraying. After spraying, lesion lengths above the inoculation point were measured fortnightly for 2 months. Thereafter, plant survival was assessed after a month, then bimonthly. Plants were recorded as dead if the leaves on the inoculated stem were brown and crispy above the inoculation point.

In the second trial, the presence or absence of a lesion was noted on 0, 5 and 30 days after spraying. Plant survival was assessed in May and November 1999, and January and June 2000.

4.2.6 Environmental conditions and water relations

Monthly maximum and minimum temperatures for the time period between inoculation and the final harvest were obtained from the Bureau of Meteorology, Karnet Station No. 009111. Monthly rainfall data was provided by Alcoa World Alumina Australia from the Jarrahdale Mine.

The water relations of six uninoculated and unsprayed plants were measured in February 1999 (summer) (Figure 4.1). Pre-dawn and midday xylem pressure potentials were measured as described in Section 3.2.8.

4.2.7 Harvesting for *P. cinnamomi* extension/viability and macroscopic examination of stems

To assess the extent of colonisation by *P. cinnamomi* before phosphite treatment, six untreated inoculated plants were harvested 3 days after inoculation in the second trial, and six plants were harvested on the day of spraying in the first and second trials. After each harvest, recovered Miracloth discs were plated onto NARPH. In the absence of a visible lesion, stems were cut into 1 cm segments up to 8 cm above and below the inoculation point. Where a lesion was visible, 1 cm segments were cut up to 8 cm beyond the lesion, in addition to two 1 cm sections into the lesion. All sections were cut in half longitudinally and plated sequentially. Plates were incubated and examined as described in Section 3.2.6.

To monitor the viability of the pathogen in the first trial, 24 plants treated with 0, 2.5 or 5 g phosphite/L (six replicates/phosphite treatment plus six uninoculated plants) were harvested approximately 2 months after phosphite treatment (February 1999), and live plants with lesions (19) and six uninoculated plants were harvested 7 months after inoculation (July 1999). The remaining live plants in which the bark obscured the lesions were harvested in June 2000 (Figure 4.1). In the second trial, dead plants (six) were harvested in January 2000 and live plants with lesions (ten) were harvested in June 2000 (Figure 4.1). Of the remaining plants that had no recorded lesion, six replicates from each treatment were selected randomly and harvested in June 2000. For each plant, two 1 cm segments of lesioned stem (or segments just above the inoculation point in non-lesioned plants) were cut and plated onto NARPH, and the plates were incubated and examined as described in Section 3.2.6. For plants harvested in June 2000, the stem segments were removed from the plates in July 2000, cut into small pieces and soaked in deionised water for 72 hours at 4°C and then a further 24 hours at 20°C. The water was replaced daily to prevent the inhibition of *P. cinnamomi* by leached phenolics. After

the 4 days of soaking, the stem pieces were lightly dried with a paper towel and plated onto NARPH. Plates were incubated and examined as described in Section 3.2.6.

Plants harvested in July 1999, January and June 2000 were prepared for macroscopic examination of stems. Stems were cut transversely with a bandsaw into 1 cm segments from the inoculation point up to the lesion front. In the absence of a visible lesion, only one segment incorporating the inoculation point was cut. After cutting, each segment was sanded to remove the surface discolouration caused by the bandsaw. The extent of tissue damage, sloughing of lesioned bark, and kino vein and woundwood formation were examined.

4.2.8 Statistical analysis

In the first trial, plants for which the bark obscured lesion visibility were not included in the calculations of lesion extension, but were included in the mortality counts.

Lesion length on the day of spraying and at 50 days after spraying was tested by ANOVA to determine whether there was any significant difference in lesion lengths between plants allocated to different phosphite treatments. The lesion extension after spraying was calculated by subtracting the lesion length measured on the day of spraying from each subsequent lesion measurement. After square-root transformation to satisfy the assumptions for a normal distribution of the data, the cumulative lesion extension data at 50 days after spraying was analysed by ANCOVA to test for an effect of phosphite treatment on lesion extension. The block effect was included in the calculation of the error term, and stem diameter was a covariate.

In both trials, the percentage of dead plants was calculated for each assessment of plant mortality, and the 95% confidence interval was used to determine if there was a significant difference between phosphite treatments.

4.3 Results

4.3.1 Environmental conditions and water relations

During the summer in which *E. marginata* were inoculated with *P. cinnamomi*, the rainfall totalled 53 mm, in comparison with 155 mm rainfall during the following summer (Figure 4.1). The average monthly maximum temperature peaked in February 1999 at 33.2°C. High rainfall in January 2000 coincided with average monthly maximum and minimum temperatures of 28.9 and 16.1, respectively (Figure 4.1).

The mean pre-dawn and midday xylem pressure potentials recorded in February 1999 were -1.2 ± 0.2 and -2.5 ± 0.1 MPa.

4.3.2 Lesions and plant survival

In the first trial, lesions were extensive on the day of spraying, with a mean lesion length of 63.7 ± 5.3 mm across all treatments. Stems of 37% of plants were girdled on day 0 (six, five and eight plants allocated to the 0, 2.5 and 5 g phosphite/L treatments, respectively). There was no significant difference (P=0.2) in the lesion lengths between plants allocated to different phosphite treatments on day 0. Phosphite treatment reduced (P=0.05) post-treatment lesion extension (Figure 4.2b), but the total lesion length at 50 days after spraying was not significantly (P=0.9) affected by phosphite treatment (Figure 4.2a).

In the second trial, a total of 16 plants developed a lesion after inoculation with *P. cinnamomi*, with six, five and five individuals in the 0, 2.5 and 5 g phosphite/L treatments, respectively. Thorough examination of 18 plants recorded as non-lesioned confirmed the absence of a lesion.

Plant mortality was not significantly (P=0.05) affected by phosphite treatment 14 days after inoculation (Figure 4.3a). Every plant that was girdled on the day of

spraying died within 6 months of phosphite treatment. Of all the plants that died in the first trial, 55, 45 and 89% were girdled on the day of spraying with 0, 2.5 and 5 g phosphite/L, respectively. In contrast, girdling was not observed at day 0 in the second trial. Plants treated with 2.5 and 5 g phosphite/L 8 days after inoculation had significantly (P=0.05) less mortality within 10 months of treatment (zero deaths) than plants treated with zero phosphite (six plants dead) (Figure 4.3b).

4.3.3 Viability of *Phytophthora cinnamomi*

In the first trial, *P. cinnamomi* was recovered from 83% of Miracloth discs and 100% of inoculated plants harvested on the day of spraying. At 2 months after phosphite treatment, *P. cinnamomi* was not recovered from any of the Miracloth discs. Despite the appearance of extensive stem lesions on all inoculated plants, *P. cinnamomi* was isolated from just two plants, both of which had received the 5 g phosphite/L treatment. In subsequent harvests, no *P. cinnamomi* was isolated from inoculated stems.

In the second trial, *P. cinnamomi* was recovered from 83% of Miracloth discs and was isolated from 66% of plants harvested 5 days before spraying. There was no recovery or isolation of *P. cinnamomi* from the Miracloth discs or from the six plants harvested on the day of spraying. *P. cinnamomi* was not isolated from inoculated stems in subsequent harvests.

P. cinnamomi was not isolated from uninoculated Miracloth discs or plants.

4.3.4 Macroscopic observations

There was no difference between the 0, 2.5 or 5 g phosphite/L treatments or between the first and second trials in the extent of tissue damage, sloughing of lesioned bark, or kino vein and woundwood formation when the lesioned stems were examined macroscopically. Lesions extended through the bark tissue and into the

xylem in 63% of lesioned samples, with up to 75% of the xylem area discoloured in some samples (Figure 4.4a). Between 5 and 100% of the vascular cambium was damaged in the segment incorporating the inoculation point. In 85% of the samples, kino veins were produced towards the xylem from the healthy vascular cambium. The kino veins extended from the segment incorporating the inoculation point to beyond the lesion, and ranged from scattered pockets to a band all around the stem (Figure 4.4b). After kino vein formation, the vascular cambium resumed the production of xylem tissue. In some samples with extensive tissue damage a large growth of woundwood on the healthy side of the stem was observed (Figure 4.4c). The new xylem terminated as curls of woundwood at the junction of healthy and lesioned tissue. Clumps of woundwood also formed as islands in healthy bark tissue. New xylem production generally became progressively more organised and normal with increasing distance from the inoculation point.

Bark lesions were in the process of being sloughed off in at least ten samples. The most common area of detachment was near the inoculation point. The loss of damaged bark occasionally left the xylem surface exposed (Figure 4.4d). Some segments of lesioned bark were so loosely attached to the stem that they fell off during sample preparation, while others were just starting to peel away from the stem (Figure 4.4e). Two plants from each phosphite treatment were severely damaged and breaking apart at the inoculation point (Figure 4.4f).

Plants that had died prior to the harvest exhibited similar characteristics to the live plants, with the sloughing off of lesioned bark, and kino vein and woundwood formation. Neither kino veins nor woundwood were observed in uninoculated plants or in inoculated plants that had no lesion.

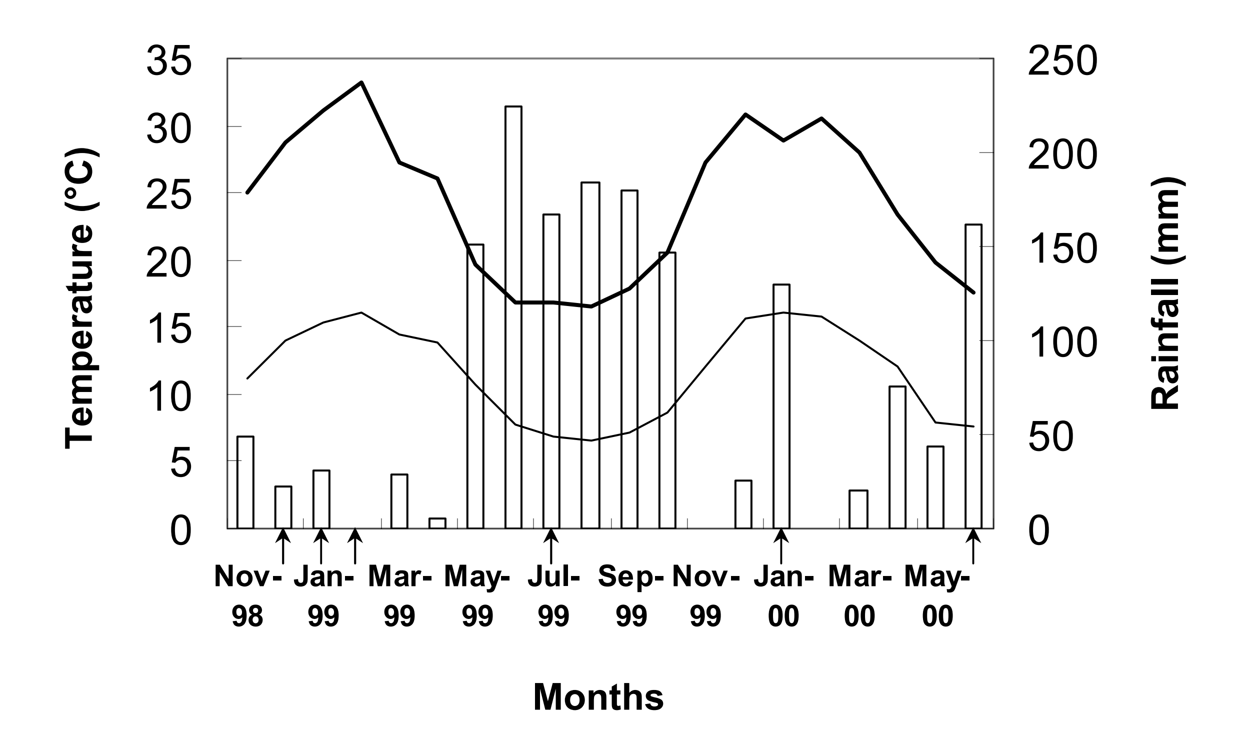
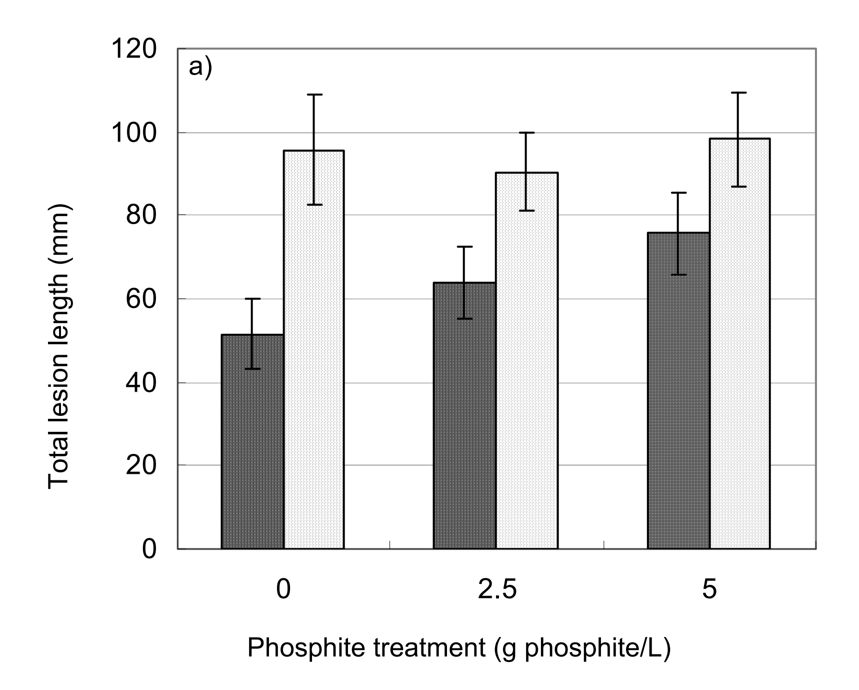


Figure 4.1: Monthly maximum (▬) and minimum (—) temperatures at Karnet and monthly rainfall (□) at Jarrahdale after inoculation of *Eucalyptus marginata* with *Phytophthora cinnamomi* and until plants were harvested. Arrows below the x-axis indicate (from left to right):

1. Trial 1 – inoculation and phosphite treatment
2. Trial 2 – inoculation
3. Trial 1 – first harvest

   Trial 2 – phosphite treatment

   Trials 1 and 2 – water relations
4. Trial 1 – second harvest
5. Trial 2 – first harvest
6. Trials 1 and 2 – final harvest



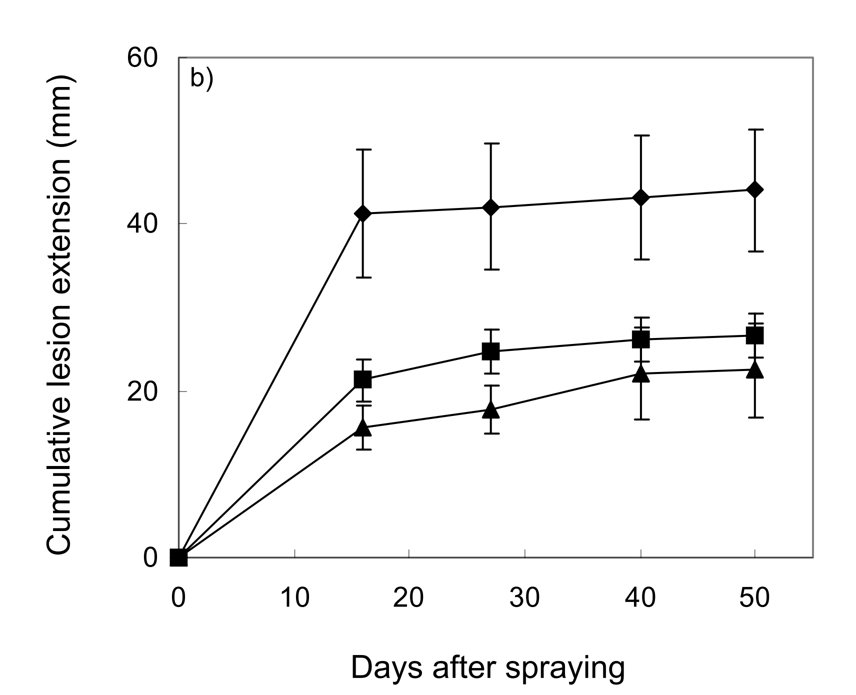


Figure 4.2: a) Total lesion length in *Eucalyptus marginata* seedlings at 0 (▩) and 50 (▨) days after spraying, and b) lesion extension after spraying foliage with 0 (◆), 2.5 (■) and 5 (▲) g phosphite/L. Plants were sprayed with phosphite at 14 days after inoculation with *Phytophthora cinnamomi*. Values are means of 17 plants ± the standard errors of the means.

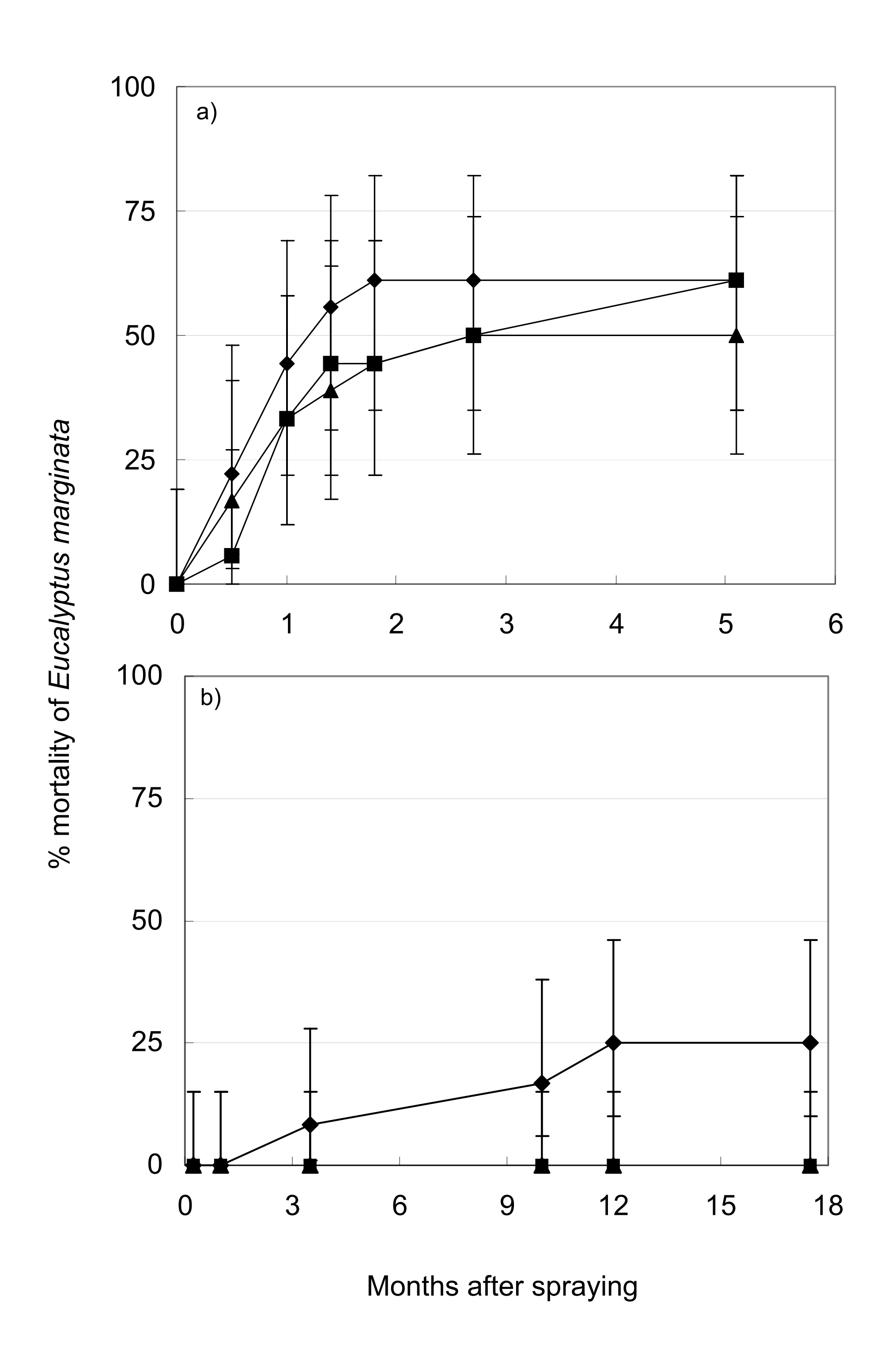


Figure 4.3: The mortality of *Eucalyptus marginata* seedlings up to a) 5 months and b) 17 months after the foliage was sprayed with 0 (◆), 2.5 (■) and 5 (▲) g phosphite/L at a) 14 days and b) 8 days after inoculation with *Phytophthora cinnamomi*. Plants were sprayed in a) December 1998 (Trial 1) and b) January 1999 (Trial 2). Bars represent the 95% confidence interval for each value [a): n=18 and b): n=24)].

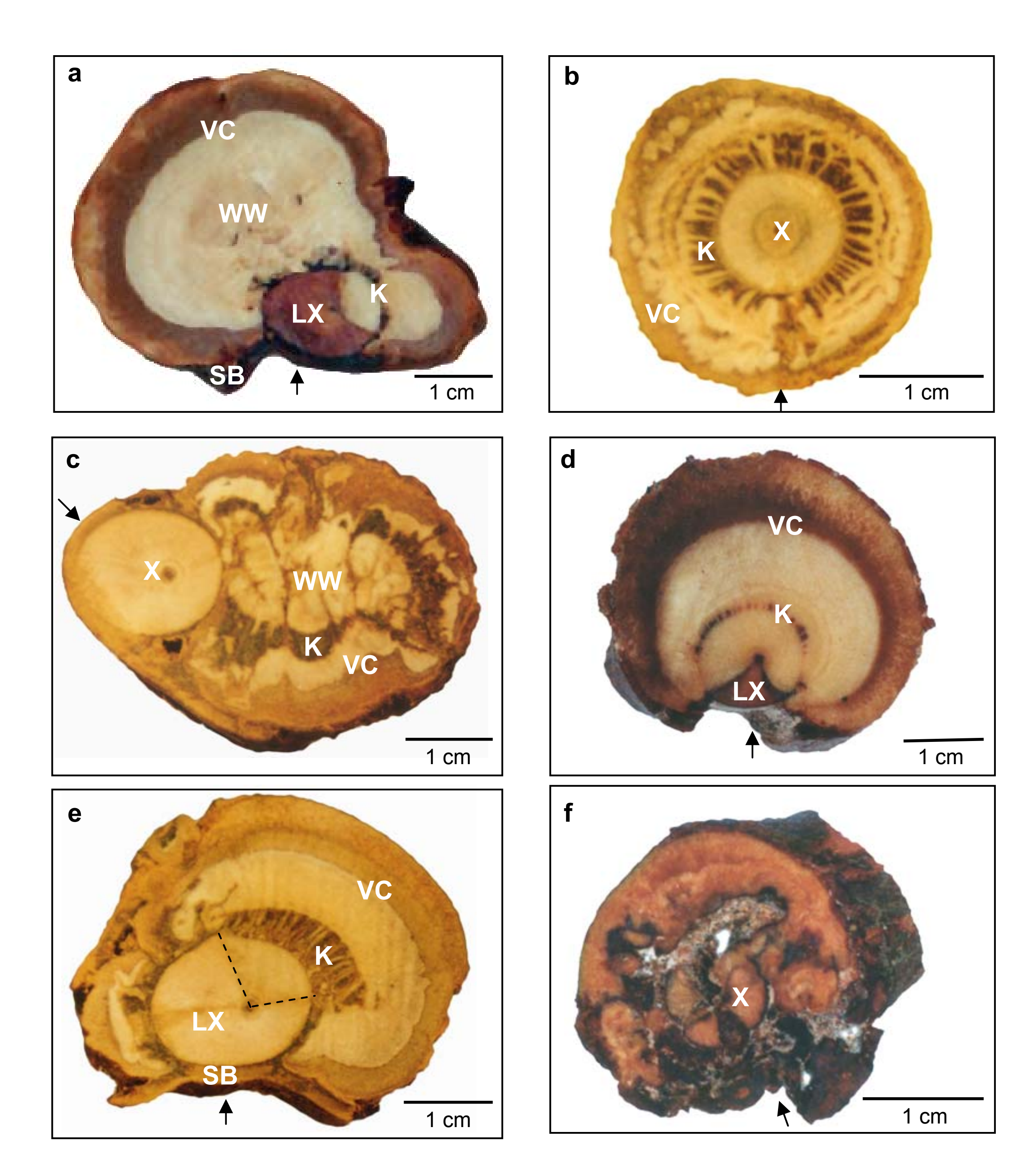


Figure 4.4: Macroscopic responses of *Eucalyptus marginata* stems to colonisation by *Phytophthora cinnamomi*. **a+b** were treated with 0 g phosphite/L, while c-f received the 5 g phosphite/L treatment. **a**: Lesioned xylem (LX), kino vein (K) and woundwood (WW) formation at 4 cm away from the inoculation point (IP). **b**: Extensive formation of kino veins at 5 cm away from the IP. **c**: Disorganised woundwood and kino vein formation, and extensive damage to vascular cambium at the IP. **d**: Exposure of xylem where the lesioned bark has been sloughed off at the IP. Also kino vein formation and lesioned xylem. **e**: Lesioned bark ready to be sloughed off at 5 cm away from the IP. Lesioned xylem is outlined by the dotted line. Also kino vein formation. **f**: Stem breaking apart at the IP. Further abbreviations (for anything not mentioned previously in text): SB=sloughed bark, VC=vascular cambium, and X=xylem. Arrows indicate the side of inoculation.

4.4 Discussion

Phosphite was ineffective at protecting one-year-old *E. marginata* seedlings from *P. cinnamomi* once the pathogen was well established in the plant, which agrees with the findings of Greenhalgh *et al*. (1994) and Aberton *et al*. (1999). It differs, however, from the report by Pegg *et al.* (1985) on the effective control of *P. cinnamomi* in diseased avocado trees. In avocado, *P. cinnamomi* attacks the fine feeder roots, and phosphite-treated plants have the capacity to replace damaged roots (Pegg *et al*., 1990). The situation was quite different in the current study, where the main stem was infected to simulate collar rot. The focus of the current study was not whether the main stem could be replaced (for example, with epicormic shoots), but whether phosphite could provide *E. marginata* with a mechanism to overcome damage within the main stem. Although girdling has been linked with the death of *Banksia* spp. infected with *P. cinnamomi* (McCredie *et al.*, 1985), seedlings of *E. regnans* and *E. viminalis* have been shown to survive when the stem is mechanically bark-girdled (Wilson and Bachelard, 1975). This suggests that girdling does not inevitably lead to the death of plants. In the current study, however, all plants that were girdled at the time of phosphite treatment eventually died, regardless of phosphite treatment.

Macroscopic observations of infected *E. marginata* stems showed a remarkable ability of the plants to replace damaged tissues, provided there was still sufficient live tissue to generate new cells. The responses were much more dynamic than those seen previously in *E. marginata* (Tippett *et al*., 1983) and *Quercus rubra* (Robin *et al*., 1992) infected with *P. cinnamomi*. The occurrence of isolated nests of xylem within the bark has been observed previously in *E. regnans* in response to injury (Cremer, 1963). In the current study, similar responses were observed in most plants that remained alive up to 17 months after inoculation, independent of phosphite treatment. Presumably, these plants initially possessed a

certain degree of resistance to *P. cinnamomi* since they were not girdled on the day of spraying. However, the occurrence of similar responses in dead plants suggests that the observed responses were not effective in the containment of *P. cinnamomi*. The production of kino by infected *E. marginata* has previously been reported to be ineffective against *P. cinnamomi* (Tippett *et al*., 1983). The production of woundwood was related to wound closure and regeneration of tissues, which are important in the recovery of plants from damage but not in restricting movement of the pathogen (Shigo, 1989). The sloughing of diseased tissues suggests some containment of the pathogen, as noted by Biggs (1993) with canker fungi. A brief microscopic examination of stems from the current study showed the presence of a wound periderm forming at the edge of lesions (data not presented). Since wound periderms have been proposed to be important in the *E. marginata*-*P. cinnamomi* interaction (Tippett and Hill, 1984), the effect of phosphite on the formation of wound periderms will be investigated further in subsequent chapters.

The effect of phosphite on the viability of the pathogen could not be tested since *P. cinnamomi* was rarely isolated from untreated plants. *P. cinnamomi* has been reported to become more difficult to isolate with increasing time after inoculation (Tippett *et al*., 1983; O'Gara, 1998). Tissue-washing has recently been suggested as a method to increase positive isolations of *P. cinnamomi* from *E. marginata*, possibly by removing inhibitory phenolics (Hüberli *et al*., 2000). It is possible that *P. cinnamomi* was still viable in *E. marginata* in the current study but was not detected with the isolation method used.

Conditions in the second trial, when *P. cinnamomi* was not well established in *E. marginata*, were not ideal to test the hypothesis. Plants were water-stressed at 5 days prior to inoculation, with the pre-dawn xylem pressure potential indicating a level of water stress that has been reported to restrict the growth of *P. cinnamomi* in *E. marginata* (Tippett *et al*., 1987). The inoculum also appeared to have low

viability, since *P. cinnamomi* could not be isolated from Miracloth discs or plants within 8 days of inoculation. Very few untreated plants died in comparison with the first trial. Despite the problems encountered, all of the untreated lesioned plants died and all sprayed lesioned plants survived after conditions ideal for *P. cinnamomi* (warm weather and summer rainfall). This suggests that phosphite has the capacity to protect *E. marginata* from *P. cinnamomi*. However, some of the sprayed plants were severely damaged and unlikely to survive any further stress (such as water stress).

Although field trials reflect the forest environment more closely than glasshouse trials, a major disadvantage is the lack of control over many variables (such as environmental conditions and plant size). The following chapters will examine the post-inoculation efficacy of phosphite in detail under more controlled conditions.

Chapter 5: Effect of phosphite on disease development and histological responses of *Eucalyptus marginata* to abiotic wounding and colonisation by *Phytophthora cinnamomi* under glasshouse conditions

5.1 Introduction

In Chapter 4, phosphite was shown to have some potential for protecting *E. marginata* from damage by *P. cinnamomi* when applied post-inoculation in a rehabilitated mine area. It was decided to conduct a more detailed experiment in the glasshouse to investigate the post-inoculation efficacy of phosphite under more controlled conditions.

Glasshouse studies on the efficacy of phosphite have found that it is not effective at controlling disease when applied greater than 24 hours after infecting tomato plants with *P. nicotianae* (Flett *et al*., 1990), or ten days after inoculating *Leucadendron* hybrids with *P. cinnamomi* (Marks and Smith, 1992). The latter report highlighted that phosphite was not effective at post-inoculation disease control in a highly susceptible species. In contrast, Wilkinson *et al*. (2001b) reported that phosphite was effective at disease control when applied two days after inoculation of the highly susceptible *Banksia grandis* and *B. hookeriana* with *P. cinnamomi*.

Several studies have reported a stimulation of defence mechanisms in response to the infection of plants treated with phosphite or Fosetyl-Al. The majority of these have examined the effect of phosphite (or Fosetyl-Al) on early defence responses, such as hypersensitive cell death (Guest, 1984; Guest, 1986), the induction of host defence enzymes (Nemestothy and Guest, 1990; Jackson *et al.*,

2000), and the synthesis and accumulation of phenolics (Khan *et al*., 1986; Jackson *et al*., 2000) and phytoalexins (Guest, 1984; Khan *et al*., 1986; Saindrenan *et al*., 1988a; Afek and Sztejnberg, 1989; Nemestothy and Guest, 1990). The effect of phosphite on delayed responses has received little attention, despite the existence of an extensive body of literature discussing the importance of histochemical and anatomical barriers as defence mechanisms against a range of pathogens (see Literature Review, Chapter 1). *P. cinnamomi* was observed to be successfully compartmentalised by reaction zones in the highly susceptible *Banksia brownii* after treatment with phosphite (Smith *et al*., 1997). Marks and Smith (1992) noted that a wound periderm confined *P. cinnamomi* to cortical tissue in *Leucodendron* hybrids protected by phosphite.

In the present study, three experiments were conducted to achieve the following aims: 1) to determine the effect of post-inoculation phosphite treatment on a) lesion extension by *P. cinnamomi*, b) plant mortality in clonal lines of *E. marginata* with known differences in susceptibility to *P. cinnamomi*, and c) histological responses of clonal *E. marginata* to *P. cinnamomi*; and 2) to determine the effect of phosphite on the wound responses of *E. marginata* in the absence of the pathogen.

5.2 Materials and methods

5.2.1 Experimental design

Experiment 1: Colonisation by *P. cinnamomi* preliminary trial

This experiment was conducted to assist the development of protocols in the following experiment. The independent variable was foliar treatment of *E. marginata* ramets [susceptible clonal line (SS) – details given in Section 5.2.2] with 0 and 5 g phosphite/L at 5 days after inoculation with *P. cinnamomi*. The dependent variables were the time at which lesion extension was significantly affected by phosphite treatment, the extension of *P. cinnamomi* beyond the lesion and the isolation of

P. cinnamomi from the plants at 8, 16 and 32 days after inoculation. The trial had a completely randomised design. There were nine replicate plants for each treatment, plus three uninoculated plants not treated with phosphite.

Experiment 2: Colonisation by *P. cinnamomi* trial

The independent variables were clonal line of *E. marginata* [resistant (RR) and SS – details given in Section 5.2.2], foliar treatment with 0, 2.5, 5 and 10 g phosphite/L at 4 days after inoculation with *P. cinnamomi*, and time after phosphite treatment (2, 4, 6, 8 and 24 days). The dependent variables were lesion extension, the extent of colonisation by *P. cinnamomi* beyond the lesion, mortality of the ramets, and the histological response of the stems to *P. cinnamomi* at the lesion front. The trial had a randomised incomplete block design. Eight (RR) and twelve (SS) replicate plants for each treatment were inoculated and monitored for 24 days after phosphite treatment. Four (RR) and six (SS) replicate plants for each treatment were harvested at 2, 4 and 6 days after phosphite treatment, while only the remaining live plants were harvested at 24 days after phosphite treatment. There were fewer replicates of the RR genotype than the SS genotype due to a shortage of RR plants. Twelve plants of each clonal line were uninoculated and not treated with phosphite.

Experiment 3: Wound responses trial

The independent variables were clonal line of *E. marginata* (RR and SS), treatment of ramets with phosphite 1 day before wounding the stem with liquid nitrogen (0 and 5 g phosphite/L in RR and 0, 5 and 10 g phosphite/L in SS), and time of harvest after wounding (7, 14 and 28 days in RR and 7-day intervals for 42 days in SS). The dependent variable was the histological response of the stems to wounding. The trial had a randomised incomplete block design. There were four and six

replicate plants for each phosphite treatment and harvest time for the RR and SS clonal lines, respectively.

5.2.2 Plant material

Ramets of the two clonal lines of *E. marginata* were provided by Marrinup Nursery (Alcoa World Alumina Australia). Screening for resistance in *E. marginata* has previously determined that clonal line 11J402 (SS) is susceptible to *P. cinnamomi* and 1J30 (RR) is resistant (McComb *et al*., 1990). The 12-16-month-old ramets were planted into 140 mm free draining pots approximately 4 months before the commencement of each experiment. The potting mix was a 2:1 mixture of peat and perlite, with the addition of nutrients (O'Gara *et al*., 1996). Three weeks before the beginning of each experiment, approximately 12 g of a low P, slow-release fertiliser (Osmocote, Scotts Australia Pty Ltd) were placed around each plant. Side shoots were removed from the plants regularly prior to the experiment. During Experiment 1, plants were hand-watered daily. During Experiments 2 and 3, plants were watered by an automatic overhead watering system, except for hand-watering in the 5 days following phosphite treatment. Plant height and diameter were not measured in Experiments 1 or 3, but plants in Experiment 2 had a mean height and diameter (10 cm above substrate level) of 46.4±0.5 and 3.4±0.04 cm, respectively. Figure 5.1 shows the plants used in Experiment 2.



Figure 5.1: Plant material and arrangement in Experiment 2.

5.2.3 Experimental conditions

The experiments were conducted in September 1997 (Experiment 1), December 1997/January 1998 (Experiment 3) and February/March 1999 (Experiment 2). Temperatures in the glasshouse were measured using a digital maximum/minimum thermometer (Rowe Scientific, Australia).

5.2.4 Inoculum material, inoculation procedure and wounding procedure

The inoculum material and inoculation procedure for Experiments 1 and 2 were the same as that described in Section 3.2.5, with the underbark inoculation of main stems with Miracloth discs colonised with *P. cinnamomi* isolate MP94-48. Sterile Miracloth discs were used for the uninoculated plants. The stems were still green at the inoculation point (approximately 10 cm above soil substrate level).

In Experiment 3, green stems of *E. marginata* were wounded at internodes approximately 10 cm above the soil substrate level. Cotton buds were dipped into liquid nitrogen and then placed against the main stem for 5 seconds. The wounded area was approximately 5 mm in length and width.

5.2.5 Spray application

Plants were treated with phosphite 5 and 4 days after inoculation with *P. cinnamomi* in Experiments 1 and 2, respectively, and 1 day prior to wounding in Experiment 3. The surface of the potting mix was covered with a woollen blanket to prevent soil drenching. The leaves were then sprayed to run-off with phosphite using a hand sprayer. The phosphite solutions were prepared from Foli-R-Fos (Unitec Group Pty Ltd, Australia), a 40% w/v solution of mono-di potassium phosphite. All treatments contained 0.25% Synertrol oil (Organic Crop Protectants Pty Ltd, Australia).

5.2.6 Monitoring

In Experiment 1, the lesion extension above and below the inoculation point was measured daily for 8 days after inoculation. Plant health was monitored daily for 32 days, and plants were recorded as dead when every leaf above the inoculation point was crispy.

In Experiment 2, the lesion extension above the inoculation point was measured daily for 11 days, then on days 13, 15, 17, 20 and 23 after inoculation. Lesion extension below the inoculation point was also measured initially, but periderms in the lower region of the stems made accurate measurements impossible. Plant mortality was recorded daily.

Plants were not assessed for phytotoxicity in Experiments 1 or 3. In Experiment 2, plants were assessed for phytotoxicity symptoms 4 days after treatment using the rating system outlined in Section 2.2.4. Phytotoxicity symptoms in plants that were harvested prior to this were assessed on the day of harvest.

5.2.7 Harvesting

Experiment 1: Colonisation by *P. cinnamomi* preliminary trial

All plants that received the zero phosphite treatment were harvested at 8 days after inoculation with *P. cinnamomi*, plus three randomly selected inoculated plants treated with 5 g phosphite/L and the three uninoculated plants. A further three plants treated with 5 g phosphite/L were harvested 8 days later, with the final three harvested after another 16 days. The first harvest time coincided with the time at which plants were starting to die above the inoculation point. The remaining plants were left longer to provide histological samples to practice with. At each harvest, a 1 cm segment of stem including 5 mm into the visible lesion and 5 mm above the lesion was sampled and fixed for histological studies (data not presented). Three 5 mm segments were then cut from the lesion, plus 5 mm segments up to 4.5 cm

above the lesion front (except in cases where the plant had been dead above the inoculation point for several days, in which the stem above the lesion was not plated). In the uninoculated plants, a 1 cm segment above the inoculation point was plated. Segments were sliced in half longitudinally and plated onto NARPH. Plates were incubated and examined as described in Section 3.2.6.

Experiment 2: Colonisation by *P. cinnamomi* trial

The sequence of harvests for Experiment 2 is summarised in Figure 5.2. Four and six replicate plants of the RR and SS genet, respectively, were harvested at 2, 4, and 6 days after treatment with phosphite. At 24 days after phosphite treatment, all the remaining live plants were harvested (n=2, 4, 5, 3, 0, 1, 1 and 3 for treatments RR/0, RR/2.5, RR/5, RR/10, SS/0, SS/2.5, SS/5 and SS/10, respectively). For each plant, a 1 cm segment of stem including 5 mm into the lesion and 5 mm above the lesion was cut using a single-edge razor blade and placed immediately into modified Karnovsky's fixative (Glauert, 1975) for histological studies. Vials were put under vacuum for 30 minutes then stored at 4°C. A 1 cm segment into the lesion was then cut, plus 1 cm segments up to 3.5 cm beyond the lesion. Segments were sliced in half longitudinally and plated onto NARPH. The remaining stem was kept at 4°C and a further 3 cm were plated if *P. cinnamomi* was isolated from all three segments. In the uninoculated plants, a 1 cm segment either side of the inoculation point was plated from three replicate plants of each genet at each harvest, and a 1 cm stem segment was cut and fixed for histological studies. Plates were incubated and examined as described in Section 3.2.6.

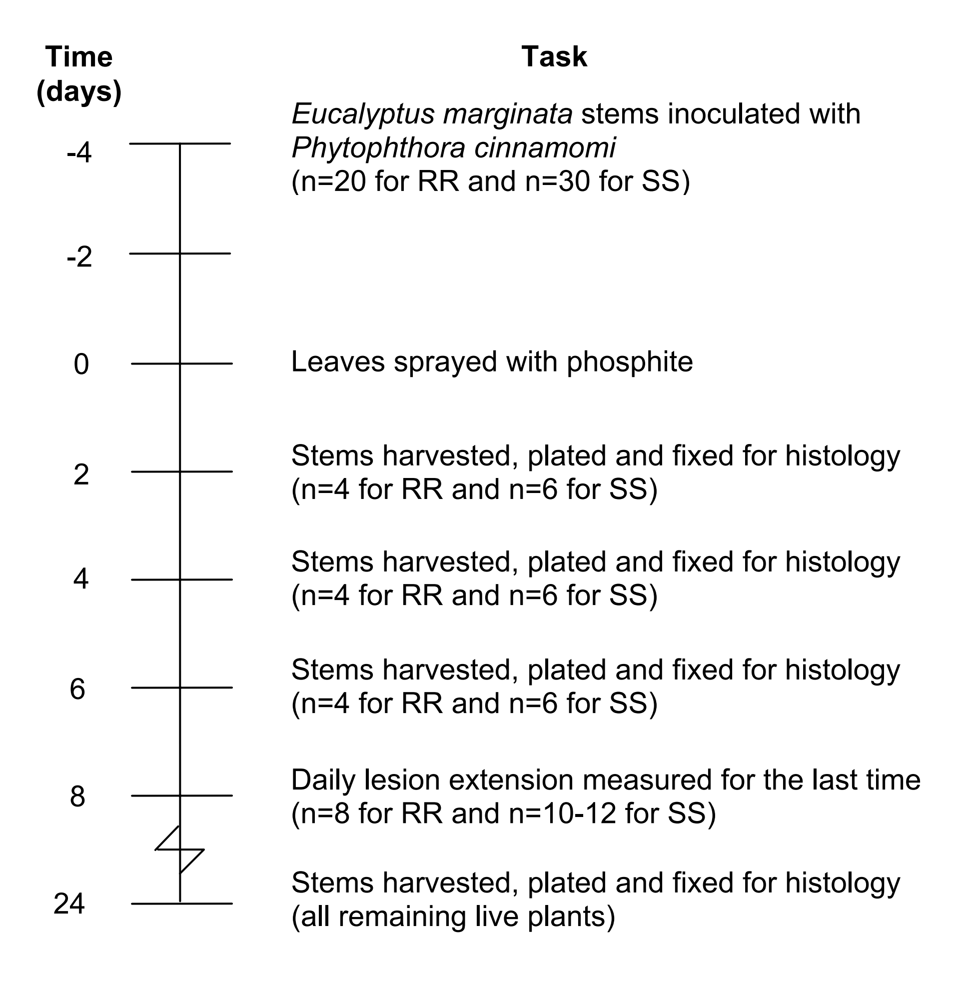


Figure 5.2: Timing of plant inoculation, phosphite treatment and harvests in Experiment 2.

Experiment 3: Wound responses trial

Four replicate plants from each phosphite treatment in the RR genet were harvested at 7, 14 and 28 days after wounding, while six replicate plants from each phosphite treatment in the SS genet were harvested at 7, 14, 21, 28, 35 and 42 days after wounding. A stem segment incorporating the wounded area was sampled and fixed in modified Karnovsky's fixative. At each harvest, a healthy segment of stem at least 4 cm away from the wounded area was cut from four replicate plants of each genet and phosphite treatment, and also placed in fixative. Vials were put under vacuum for 30 minutes then stored at 4°C.

After the examination of all samples treated with 0 g phosphite/L, two harvest times (7 and 28 days after spraying) were selected from the remaining phosphite treatments to determine the effect of phosphite on the wound response of *E. marginata*.

5.2.8 Preparation and examination of histological samples

Samples from Experiments 1 and 3 were washed three times in 0.1 M phosphate buffer approximately 1 month prior to examination. Stem segments were cut in half longitudinally to facilitate sectioning. Where possible, it was ensured that there was sufficient non-wounded/non-lesioned tissue adjacent to the wound/lesion to enable examination of the wound/lesion front. Parafilm (American National Can, United States of America) was wrapped around the stem to support the tissue and provide a guide to the thickness of sections. Transverse hand sections were cut using a single-edged razor blade. Twelve sections were cut from each sample, with four replicate sections for each stain. Samples for each stain were spread across the length of the sectioning (for example, sections two, five, eight and eleven were stained for suberin). Sections were placed into water immediately after being cut, and were stored in 2 mL Eppendorf tubes containing 0.5 mL of 0.1 M phosphate buffer.

Four sections of each sample were left unstained, and mounted in water on microscope slides. Other sections were stained with Sudan Black B (SBB) for the detection of lipids such as suberin and Phloroglucinol + HCl (PHCl) for the detection of lignin in brightfield microscopy (Biggs, 1984b, 1985a). SBB and PHCl were also used to selectively quench the autofluorescence of suberin and lignin, respectively (Biggs, 1984a, 1985a). Sections were rinsed in 70% ethanol before staining for 1 hour with SBB (0.3% in 70% ethanol), and then rinsed with 70% ethanol again for 1 minute before being stored in deionised water. Sections were mounted in water.

For PHCl, sections were stained for 4 minutes in the Phloroglucinol solution (5% in 75% ethanol) before a drop of concentrated HCl was added. Sections were then mounted in 75% glycerine (Andary *et al*., 1996), which prevented the precipitation problems encountered when mounting in Phloroglucinol. Although it is recognised that the stains do not provide conclusive evidence for the presence of suberin and lignin (Lewis and Yamamoto, 1990), the material staining positively for SBB and PHCl will generally be referred to as suberin and lignin in this thesis.

Mounted sections were examined under bright field or fluorescence. The Carl Zeiss fluorescence combination for violet light was used (consisting of exciter filter BP 390-420, chromatic beam splitter FT 425 and barrier filter LP 450). In Experiment 2, thick sections from plants harvested 24 days after phosphite treatment were examined using an Olympus dissecting photomicroscope.

5.2.9 Statistical analysis

Experiment 1: Colonisation by *P. cinnamomi* preliminary trial

Since a one-way ANOVA indicated that there was no significant difference in the lesion lengths on the day of spraying in plants allocated to the 0 and 5 g phosphite/L treatments, the lesion data was re-zeroed on the day of spraying. A one-way ANOVA was conducted on the length of lesion that developed by 2 days after spraying to determine whether the effect of phosphite treatment on lesion extension was significant. When examination of residuals indicated that these were heteroscedastic, the significance level was reset to $P=0.01$, as recommended by Tabachnik and Fidell (1996). Since a one-way ANOVA indicated there was no significant ($P>0.05$) difference between the post-spray lesion extension above and below the inoculation point, measurements from above the inoculation point only are presented.

Experiment 2: Colonisation by *P. cinnamomi* trial

Using the general linear model procedure, separate MANOVAs were conducted to test for the effect of genotype and phosphite treatment on: 1) three categories of lesion extension in non-harvested plants - pre-spray (-4 to 0 days), post-spray (0 to 8 days), and total (-4 to 8 days); 2) total lesion length in harvested plants; 3) three categories of the extension of *P. cinnamomi* beyond the lesion in harvested plants - 2, 4 and 6 days; and 4) total length of colonisation by *P. cinnamomi* in harvested plants. Block was included in the error terms. Although the analysis indicated that there was a significant (P<0.05) difference in the lesion lengths in plants allocated to different treatments on day 0, the lesion data were re-zeroed on the day of spraying to illustrate the lesion extension after spraying. For the presentation and analysis of the lesion data, five plants were removed from the dataset (with no more than two removed from any one treatment) due to the early death of four plants (4 days after phosphite treatment) and the absence of a lesion in one plant.

The extension beyond the lesion was recorded as the length of stem from the lesion front to the mid-point of the furthest centimetre from which *P. cinnamomi* was isolated. The total colonisation length was determined by adding the total lesion length to the extension beyond the lesion. The total colonisation data was combined for plants that were harvested at 2, 4 and 6 days after phosphite treatment, since an ANOVA indicated that the total colonisation length was not significantly (P>0.05) affected by the harvest time.

The percentage of dead plants in each treatment was calculated for 4-day intervals up to 24 days after spraying, and the 95% confidence interval was used to determine if there was a significant difference between phosphite treatments.

For the histological samples, the percentages of plants staining positively to PHCl and SBB in bark tissues were calculated for the first three harvests (2, 4 and 6 days after phosphite treatment). The 95% confidence interval was used to

determine if there was a significant difference in staining between the two genotypes and four phosphite treatments over time. The histological observations from the harvest at 24 days after phosphite treatment are described rather than analysed statistically.

Experiment 3: Wound responses trial

Since there was very little variation between replicate samples in the responses of *E. marginata* to wounding, the histological responses are described rather than analysed statistically.

5.3 Results

5.3.1 Experimental conditions

Temperatures in the glasshouse were similar in all three experiments, with an average maximum/minimum of 32.0/20.6°C (Table 5.1).

Table 5.1: Maximum and minimum temperatures recorded in the glasshouse during Experiments 1-3.

Experiment	Mean maximum (±SE) (°C)	Maximum range (°C)	Mean minimum (±SE) (°C)	Minimum range (°C)
1	31.8 ± 1.6	26 – 37	20.6 ± 0.7	18 – 24
2	31.7 ± 0.4	28 – 34	20.4 ± 0.8	13 – 26
3	32.5 ± 0.4	28 – 38	20.9 ± 0.4	17 – 26

5.3.2 Disease development and symptoms in *E. marginata*

5.3.2.1 Experiment 1: Colonisation by *P. cinnamomi* preliminary trial

Lesion extension in *E. marginata* was significantly (P=0.003) reduced by treatment with 5 g phosphite/L within 2 days of spraying (Figure 5.3). During this time the lesions in plants treated with 0 g phosphite/L had extended twice as far as lesions in plants receiving the 5 g phosphite/L treatment.

P. cinnamomi was isolated from all lesioned stem segments in each of the three harvests. In harvest 1, *P. cinnamomi* had colonised the stem 1 cm in advance of the lesion in five out of nine plants treated with zero phosphite, and one out of three plants treated with 5 g phosphite/L. In the other two harvests (5 g phosphite/L treatment only), *P. cinnamomi* was isolated from beyond the lesion in all six plants, with a mean extension of 2 cm beyond the lesion.

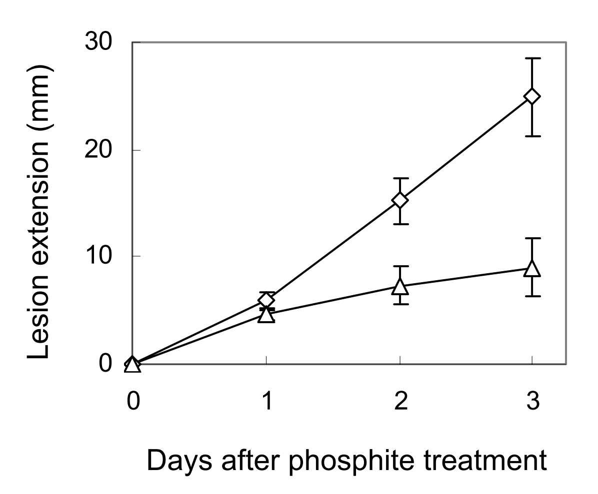


Figure 5.3: Daily cumulative lesion extension in stems of *Eucalyptus marginata* [susceptible (SS) genotype] after foliar treatment with 0 (◇) and 5 (△) g phosphite/L. Plants were inoculated with *Phytophthora cinnamomi* 5 days prior to spraying. Values are means of repeated measurements of nine plants ± the standard errors of the means.

5.3.2.2 Experiment 2: Colonisation by *P. cinnamomi* trial

Lesion extension

Lesion length on the day of spraying was significantly affected by genotype, with shorter lesions in the RR than in the SS genotype (Figure 5.4 and Table 5.2). Plants allocated to the 2.5 g phosphite/L treatment had significantly shorter lesions than those selected for the 0 and 5 g phosphite/L treatments (Figure 5.4 and Table 5.2).

Lesions continued to extend up to 7 days after treatment with 0 g phosphite/L, whereas the lesion extension had generally levelled off by 4 days after treatment with 2.5, 5 and 10 g phosphite/L (Figure 5.5). Genotype, unlike phosphite treatment, did not significantly affect post-spray lesion extension (Table 5.2). There was a significant interaction between genotype and phosphite treatment in the post-spray lesion extension, with lesions in plants receiving the 0 g phosphite/L treatment shorter in RR than in SS, but longer in RR than in SS in plants treated with 5 and 10 g phosphite/L. In plants that survived after 8 days post-spraying, mean lesion extension was 6 mm in the 0 g phosphite/L treatment and <1.5 mm in the 2.5, 5 and 10 g phosphite/L treatments.

The total lesion extension in *E. marginata* (from the day of inoculation up to 8 days after spraying) was significantly affected by genotype and phosphite treatment (Table 5.2).

Colonisation by *P. cinnamomi* beyond the lesion

P. cinnamomi was isolated from all lesioned stems. The extension of *P. cinnamomi* beyond the lesion was greater in the RR than in the SS genotype at 2 and 6 days after phosphite treatment (Figure 5.6 and Table 5.3), with a maximum mean extension of 2.6 cm in the RR/0 treatment. Phosphite treatment had no significant effect on extension beyond the lesion at any of the harvest times (Table 5.3). When the extension beyond the lesion was added to the lesion length, there was no

difference in total colonisation between the two genotypes (Table 5.3).

In the final harvest 24 days after phosphite treatment, *P. cinnamomi* extended approximately 1 cm beyond the lesion in the 2.5, 5 and 10 g phosphite/L treatments in the RR genotype. In the two live RR plants treated with 0 g phosphite/L, there were isolated lesion patches around nodes above the lesion front, and *P. cinnamomi* was isolated from 10 and 17 cm beyond the lesion front. *P. cinnamomi* was not isolated beyond the lesion in the SS/10 treatment. There were insufficient plants alive from the SS/0, SS/2.5 and SS/5 treatments to obtain data for the extension beyond the lesion.

Plant mortality

The first plant mortalities occurred in the SS genotype at 4 days after phosphite treatment. Every plant in the SS/0 treatment died within 12 days of phosphite treatment, at which time the percentage of deaths in the SS/5 and SS/10 treatments were significantly (P=0.05) less than in the SS/0 treatment (Figure 5.7). By 24 days after phosphite treatment, SS/10 was the only treatment in the SS clonal line with significantly less mortality than in the SS/0 treatment. Plants of the RR genotype treated with 0, 2.5, 5 and 10 g phosphite/L had significantly less mortality than those of the SS/0 treatment at 12 days after spraying. By day 24, mortality in the RR/0 treatment was not significantly different from the SS/0 treatment. The only treatment that resulted in significantly less mortality than in the RR/0 treatment at 24 days after spraying was RR/5 (Figure 5.7).

Phytotoxicity

Phosphite was more toxic to plants in the RR genotype than in the SS genotype (Table 5.4), with a larger leaf area burnt in the 2.5, 5 and 10 g phosphite/L treatments. RR/10 was the only treatment that led to the development of severe phytotoxicity symptoms.

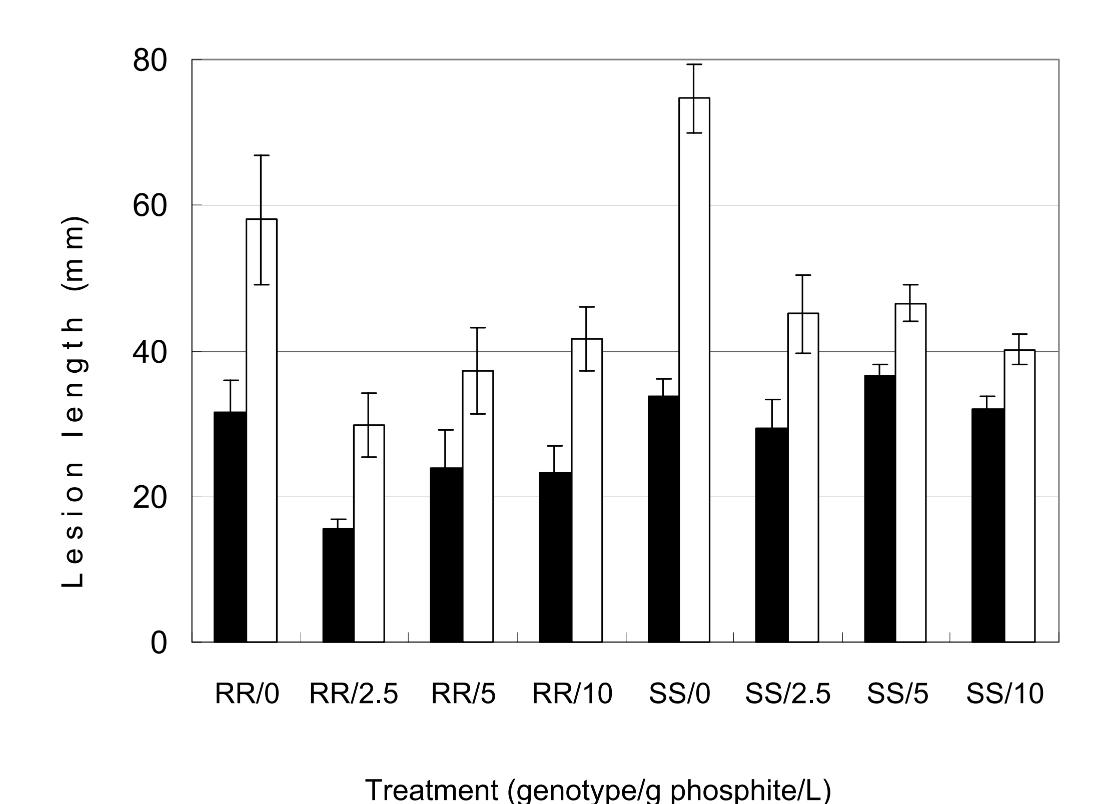


Figure 5.4: Cumulative lesion lengths above the inoculation point in stems of *Eucalyptus marginata* [resistant (RR) and susceptible (SS) genotypes] at 0 (■) and 8 (□) days after spraying the foliage with phosphite (0, 2.5, 5 and 10 g phosphite/L). Plants were inoculated with *Phytophthora cinnamomi* 4 days prior to phosphite treatment. Values are means of repeated measurements of eight to twelve plants for each treatment ± the standard errors of the means.

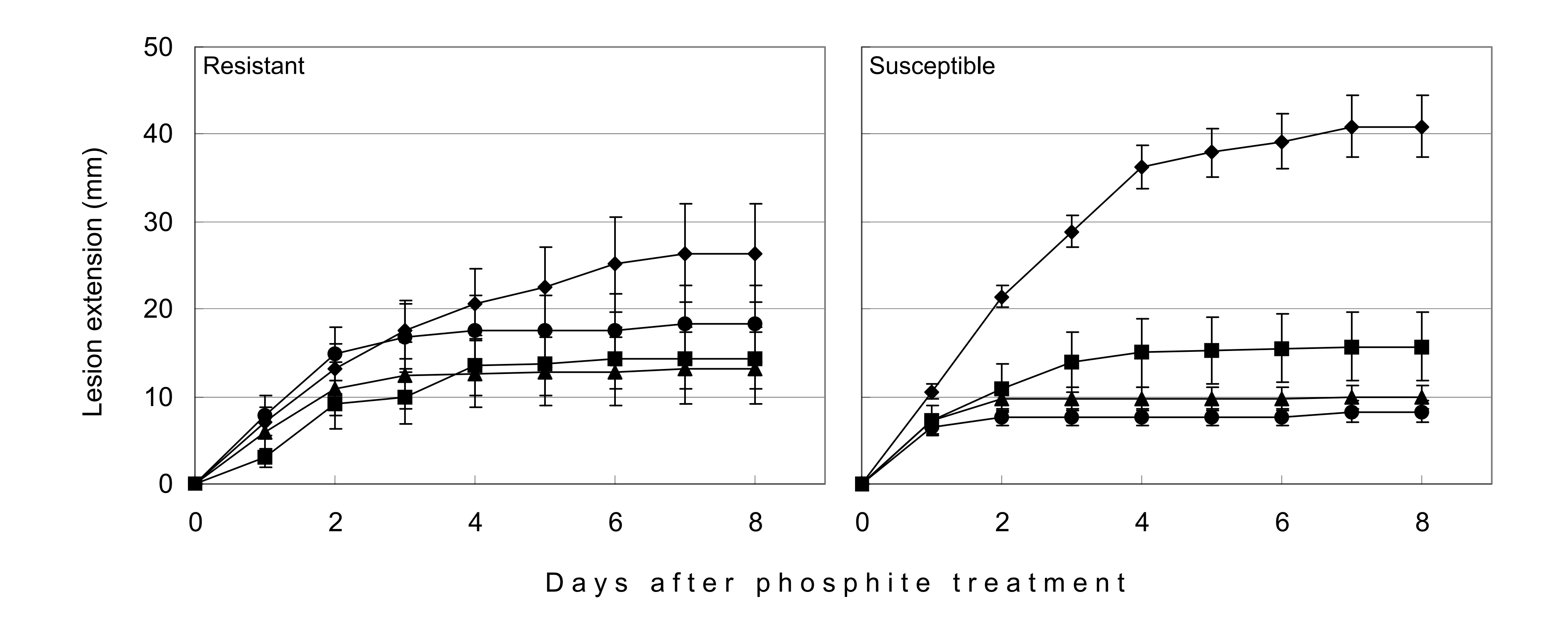


Figure 5.5: Daily cumulative lesion extension in stems of *Eucalyptus marginata* [resistant (RR) and susceptible (SS) genotypes] after foliar treatment with 0 (◆), 2.5 (■), 5 (▲) and 10 (●) g phosphite/L at 4 days after inoculation with *Phytophthora cinnamomi*. Values are means of repeated measurements of eight to twelve plants ± the standard errors of the means. Pre-spray lesion lengths have been subtracted from all means.

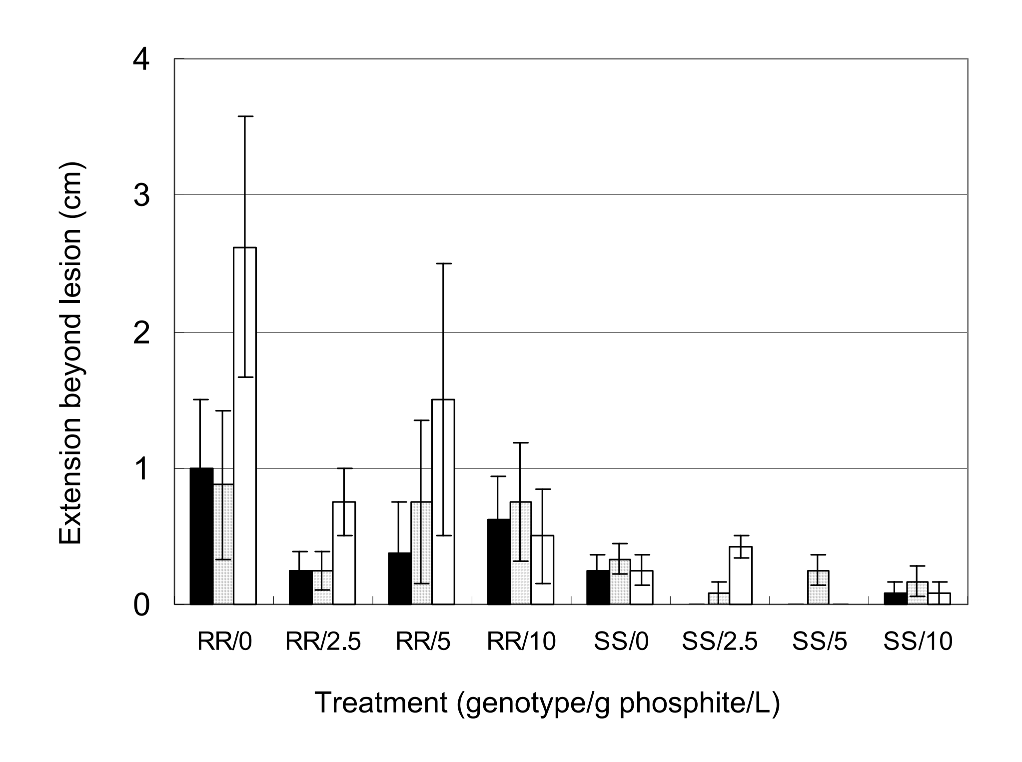


Figure 5.6: Extent of colonisation by *Phytophthora cinnamomi* beyond the lesion in stems of *Eucalyptus marginata* [resistant (RR) and susceptible (SS) genotypes] at 2 (■), 4 (▨) and 6 (□) days after spraying the foliage with 0, 2.5, 5 and 10 g phosphite/L. Values are means of four and six plants for RR and SS, respectively, ± the standard errors of the means.

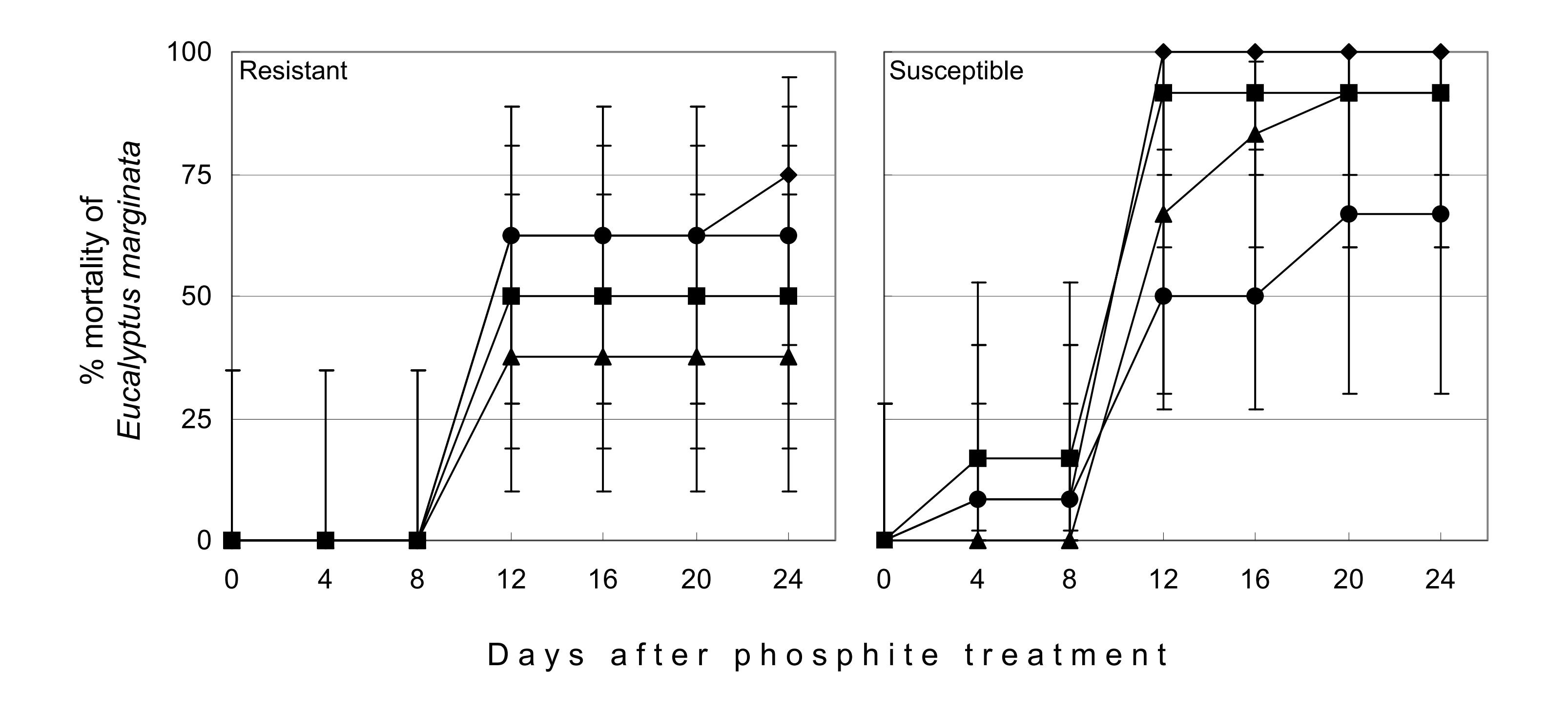


Figure 5.7: The mortality of *Eucalyptus marginata* (resistant (RR) and susceptible (SS) genotypes] up to 24 days after the foliage was sprayed with 0 (◆), 2.5 (■), 5 (▲) and 10 (●) g phosphite/L at 4 days after the stems were inoculated with *Phytophthora cinnamomi*. Bars represent the 95% confidence interval for each value (n=8-12).

Table 5.2: Summary of MANOVA[A] for the effect of plant genotype [resistant (RR) and susceptible (SS)] and phosphite treatment (0, 2.5, 5 and 10 g phosphite/L) on lesion lengths in stems of *Eucalyptus marginata*. Plants were sprayed 4 days after inoculation with *Phytophthora cinnamomi*.

Independent variable	Lesion extension (days after phosphite treatment)		
	–4 to 0	0 to 8	–4 to 8
Plant genotype	<0.001*[B]	0.98[B]	0.03*[B]
Phosphite treatment	0.02*	<0.001*	<0.001*
Genotype x phosphite	0.2	0.007*	0.3

[A] Statistical analysis of data from non-harvested plants (all data presented in Figures 5.8 and 5.9).

[B] P values from MANOVA. * indicates that effects are significant when $\alpha = 0.05$.

Table 5.3: Summary of MANOVA[A] for the effect of plant genotype [resistant (RR) and susceptible (SS)] and phosphite treatment (0, 2.5, 5 and 10 g phosphite/L) on colonisation of *Eucalyptus marginata* stems by *Phytophthora cinnamomi*. Plants were sprayed 4 days after inoculation and harvested at 2, 4 and 6 days after phosphite treatment.

Independent variable	Total lesion length	Extension beyond lesion (days after phosphite treatment)			Total colonisation length
		2	4	6	
Plant genotype	0.002*[B]	0.007*[B]	0.07[B]	0.001*[B]	0.2[B]
Phosphite treatment	0.002*	0.07	0.5	0.06	<0.001*
Genotype x phosphite	0.07	0.6	0.9	0.06	0.2

[A] Statistical analysis of data from harvested plants (extension beyond lesion is presented in Figure 5.10).

[B] P values from MANOVA. * indicates that effects are significant when $\alpha = 0.05$.

Table 5.4: Phytotoxicity ratings on leaves of the resistant (RR) and susceptible (SS) genotypes of *Eucalyptus marginata* after foliar treatment with 0, 2.5, 5 and 10 g phosphite/L.

Ratings: 0 = no burning, 1 = 1 to 25% of leaf area burnt, 2 = 26 to 50% of leaf area burnt and 3 = >50% leaf area burnt.

Phosphite treatment (g phosphite/L)	Mean phytotoxicity rating (genotype)	
	RR	SS
0	0[A]	0[A]
2.5	0.5	0
5	1.2	0.6
10	2.3	1.6

[A]Values are means of 20 plants for RR and 30 plants for SS, recorded at 4 days after phosphite treatment [except plants that were rated and harvested at 2 days after phosphite treatment (n=4 for RR and n=6 for SS)].

5.3.3 Anatomical responses in *E. marginata*

5.3.3.1 Experiment 3: Wound responses trial

Histology of healthy stems

The bark of *E. marginata* consisted of the following tissue zones: the epidermis, cortex, primary phloem and secondary phloem. Occasionally a single normal periderm was formed in the cortex. A cambial zone separated the bark from the xylem. Individual cells in the cambial zone were usually indistinct in healthy stems observed for autofluorescence. Rays were mostly one cell wide, and were continuous through the bark, cambial zone and xylem. Pith was present in the centre of the stem, but was not examined in detail. The cuticle, normal periderm (where present), phloem fibres and differentiated xylem cells exhibited blue autofluorescence when examined under ultraviolet light. When stained with PHCl, some phloem fibres and all differentiated xylem cells reacted positively with a pink/red colour. The cuticle and phellem cells of the normal periderm stained positively with SBB. No other tissues reacted positively to the stains for lignin and suberin in healthy stems.

Neither genotype nor phosphite had any effect on the histology of uninoculated/unwounded stems.

Histology of wounded stems

Placing liquid nitrogen on the stem killed all of the underlying bark. Approximately 25% of each stem's vascular cambium (VC) was damaged. *E. marginata* responded to the wound by regenerating the vascular cambium and forming periderms to isolate the wounded tissue from healthy tissue. A schematic representation of the wound responses is presented in Figure 5.8.

In the 7 days after wounding (AW), callus formed from healthy tissue adjacent to the wound (Figure 5.9d). This proliferation of cells lifted the dehydrated

wounded tissue from the xylem surface, leaving an empty space (Figure 5.9a+b). In two plants sprayed with phosphite (one in each of 5 g and 10 g phosphite/L treatments), there was insufficient callus production to lift the wounded bark away from the xylem (Figure 5.9c). By 14 days AW, callus tissue filled the previously empty space, and the continuity of the VC was almost fully restored (Figure 5.9g+h). Xylem tissue was produced internally from the new VC but differed from normal xylem in that the cells were irregularly shaped and had long pitted walls. By 21 days AW, some of the xylem produced by the new VC appeared normal, and the callus tissue internal to the VC was differentiating into lignified (PHCl +ve) woundwood (WW) (Figure 5.9e). By 42 days AW, the majority of the most recently produced xylem tissue appeared normal, and all of the callus tissue internal to the VC had differentiated into WW (Figure 5.9f). External to the VC, the production of phloem fibres was first observed at 21 days AW (from the oldest section of the new VC), and by 42 days AW, most of the length of the new VC had produced phloem fibres.

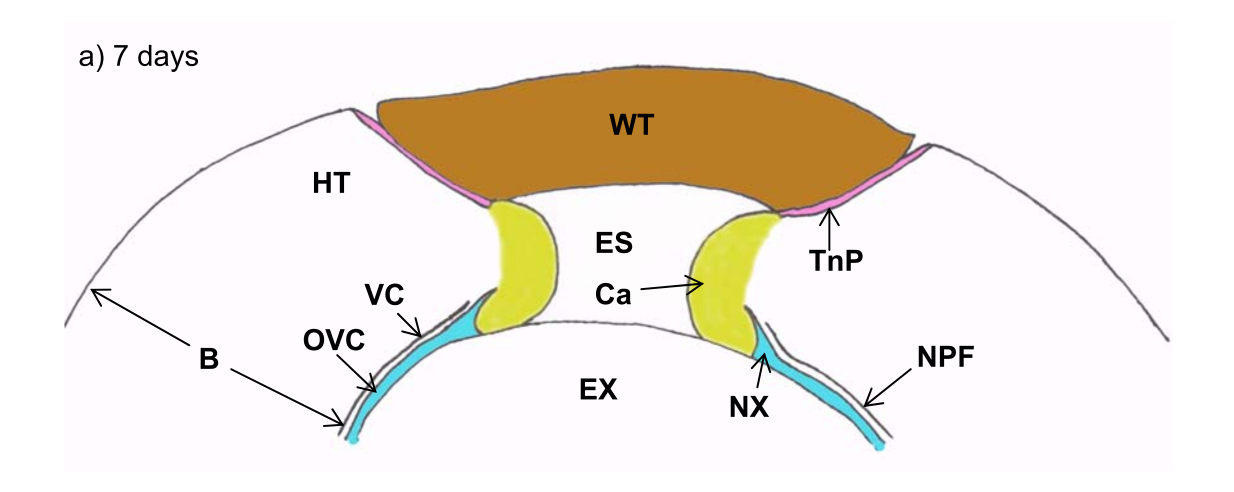
By 7 days AW, a wound periderm (WP) separated the damaged tissue from healthy tissue (Figures 5.10a+b). The WP extended along the sides of the wound, from near the cuticle to the inner wound edge. The periderm contained approximately two cell layers of thin-walled phellem (TnP), the cell walls of which stained positively for suberin (Figures 5.10c+d). Lignin was detected in the cell walls of parenchyma cells surrounding the phellem layer externally (Figures 5.10d-f). A phellogen and up to eight cell layers of phelloderm were present internal to the phellem layer. The phellogen was apparent as a layer of 'squashed' cells with obvious nuclei, and the phelloderm cells resembled normal parenchyma cells except they were in alignment with the phellogen and the suberised phellem cells of the WP.

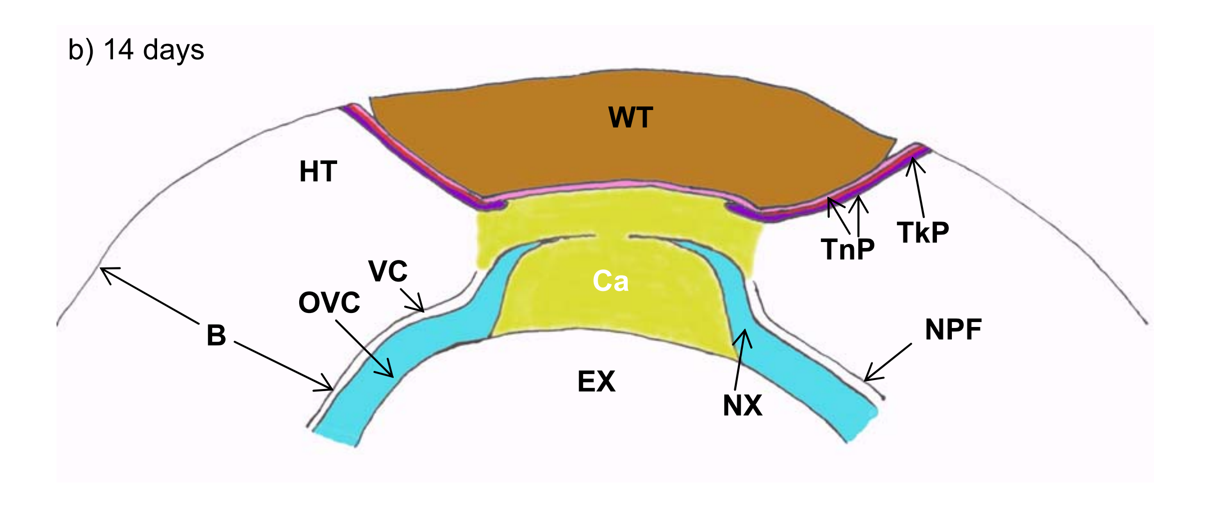
By 14 days AW, a periderm layer was formed across the entire length of the wounded/healthy tissue interface, including the outer edge of the callus tissue

(Figures 5.11a+b). No lignified cells were observed external to the periderm in the callus tissue. Towards the outer edge of the bark, a single layer of cubical TnP cells had formed in radial alignment with the first-formed phellem. This more orderly layer was internal to the first-formed phellem layers, and separated from them by up to five layers of cells in which some wall thickening (characteristic of thick-walled phellem or TkP) was evident. At this time, the results of staining the TkP with PHCl were inconclusive. The TnP layer petered out into a phellogen towards the centre of the internal wound edge (Figure 5.11c).

At 21 days AW, a layer of cubical TnP was continuous around the wound edge, and the previous phellem layer usually became distorted (Figures 5.11d+e). There were multiple cubical TnP layers at the outer edge of the wound from day 21, and it became increasingly difficult to count the exact number of phellem layers as the wound tissue continued to break away and older phellem cells were obliterated. Most of the cubical TnP layers were short and did not extend to the centre of the wound edge. Wall thickening of cells between TnP layers was also noted at 21 days AW but lignin was still not detected. By 28 days AW, TkP with lignified cell walls was observed in all samples, usually near the breaking edge of the wound (Figures 5.11g-i). The bright autofluorescence of the pitted, TkP cell walls was quenched in sections stained for lignin. The number of cell layers in the TkP ranged from two to five. Occasionally, a wedge of TkP was observed between a TnP layer and a phellogen with no apparent suberised TnP (Figure 5.11h). In addition to TkP, a pale-pink autofluorescent substance was observed at the breaking edge of the wound by 21 days AW (Figure 5.11f). This deposit was found to be suberised (Figure 5.11g), and resembled cuticle.

With the exception of the reduced callus production observed in two plants treated with phosphite, the chemical had no effect on the wound responses of *E. marginata*. There was also no genotypic effect on the wound responses.


a) 7 days
WT
HT
ES
Ca
TnP
VC
OVC
B
EX
NX
NPF


b) 14 days
WT
HT
TnP
TkP
Ca
VC
OVC
B
EX
NX
NPF

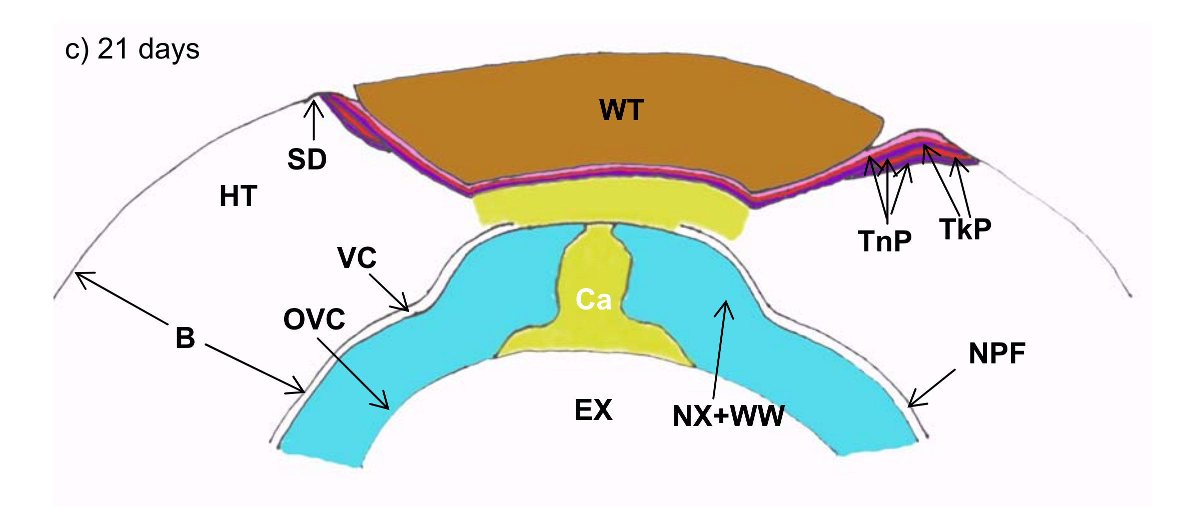

c) 21 days
WT
SD
HT
TnP
TkP
VC
Ca
OVC
B
EX
NX+WW
NPF

Figure 5.8: Schematic diagram of the wound response in stems of *Eucalyptus marginata* at a) 7, b) 14 and c) 21 days after inflicting a deep wound with liquid nitrogen.

a) The wounded bark tissue (shown in brown - WT) is lifted by the formation of callus (shown in yellow - Ca) from adjacent healthy tissue, leaving an empty space (ES) external to the xylem existing before injury (EX). Some xylem tissue has formed since the injury (shown in blue - NX). Thin-walled phellem (shown in pink - TnP) is produced by the phellogen of a wound periderm between the healthy tissue (HT) and the WT. Lignin is deposited in the walls of cells external to the TnP (not shown).

b) At 14 days after wounding, callus has extended to fill the region external to the original xylem. Some of the callus has differentiated into vascular cambium (VC), which has almost regained its continuity. Some of the regenerated VC has produced xylem (NX). A continuous layer of TnP has formed across the entire length of the WT/HT interface. A layer of TnP consisting of more orderly cubical cells (shown in purple) is visible internal to the first-formed TnP observed at 7 days after wounding. Layers of thick-walled phellem cells (TkP) separate the two TnP layers (shown in red).

c) The VC is continuous by 21 days after wounding, and most of its length has generated NX and phloem fibres (NPF). Some of the callus internal to the vascular cambium is differentiating into woundwood (WW). There is at least one continuous layer of cubicle TnP across the junction of healthy and wounded tissue, and multiple layers at the outer edge of healthy tissue. Wedges of TkP occur between phellem layers. Suberin deposits (SD) are visible in the HT near the breaking edge of the wound.

Further abbreviations (for anything not mentioned previously in text): B = bark, OVC = original VC.

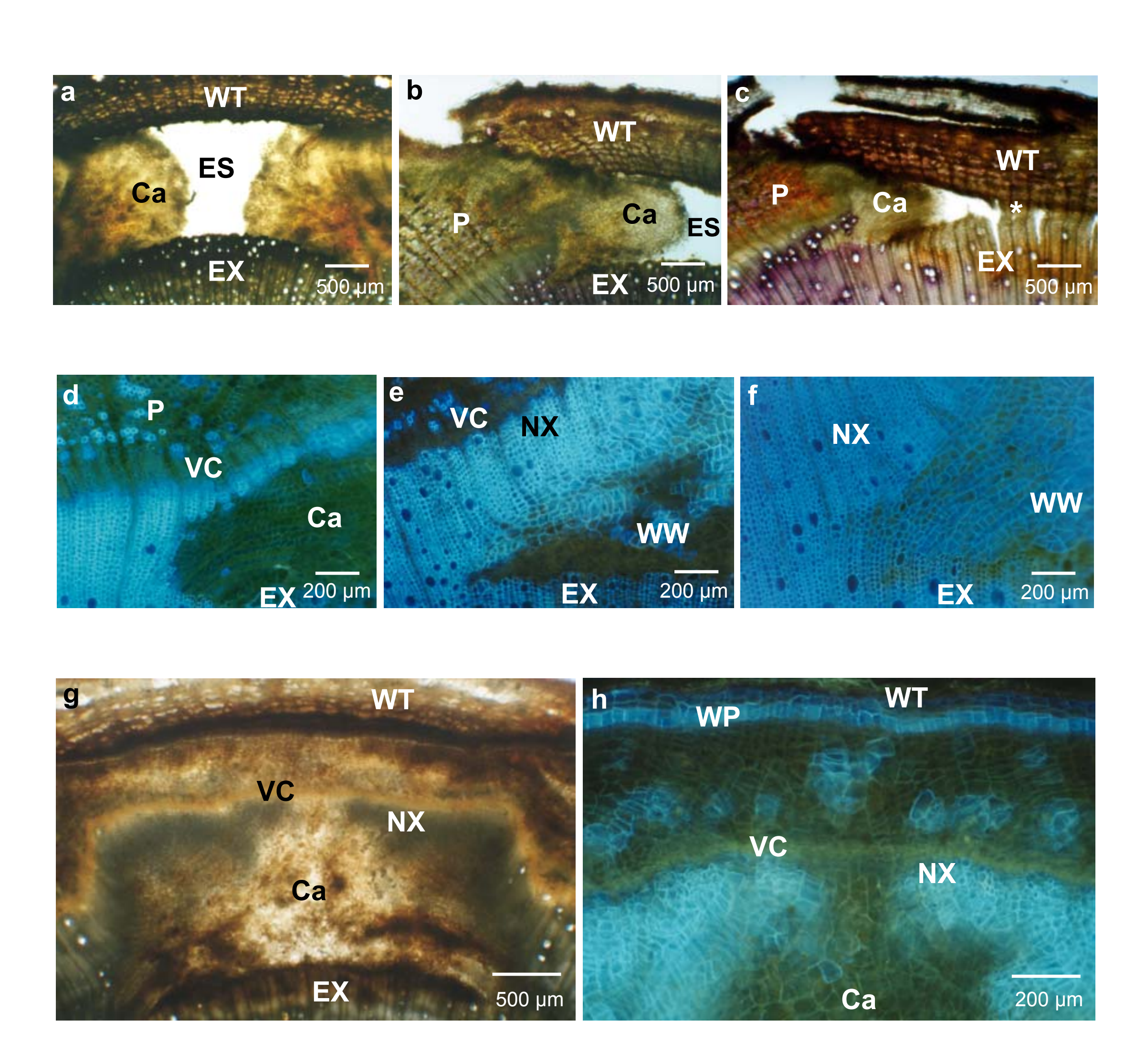


Figure 5.9: Regeneration of vascular cambium and xylem formation in clonal *Eucalyptus marginata* after inflicting a deep wound with liquid nitrogen.
a+b: Wounded tissue (WT) is lifted away from the xylem (EX) by the production of callus (Ca) at 7 days after wounding (AW) and treatment with 0 g phosphite/L.
c: Limited callus production in a plant treated with 5 g phosphite/L. Wounded tissue is still attached to the xylem (indicated by an asterisk) at 7 days AW.
d-f: Xylem formation internal to the wounded tissue at 7 (**d**), 21 (**e**) and 35 (**f**) days AW. **d:** No autofluorescence is visible in the callus. **e**: Some of the callus has differentiated into woundwood (WW) and the regenerated vascular cambium (VC) has produced some xylem (NX). **f:** Most of the callus has differentiated into woundwood. **g+h:** A completely continuous VC at 21 days AW. Most of the regenerated VC has produced NX, though the new cells have an irregular shape.

Further abbreviations (for anything not previously mentioned in text): ES = empty space, P = phloem and WP = wound periderm.

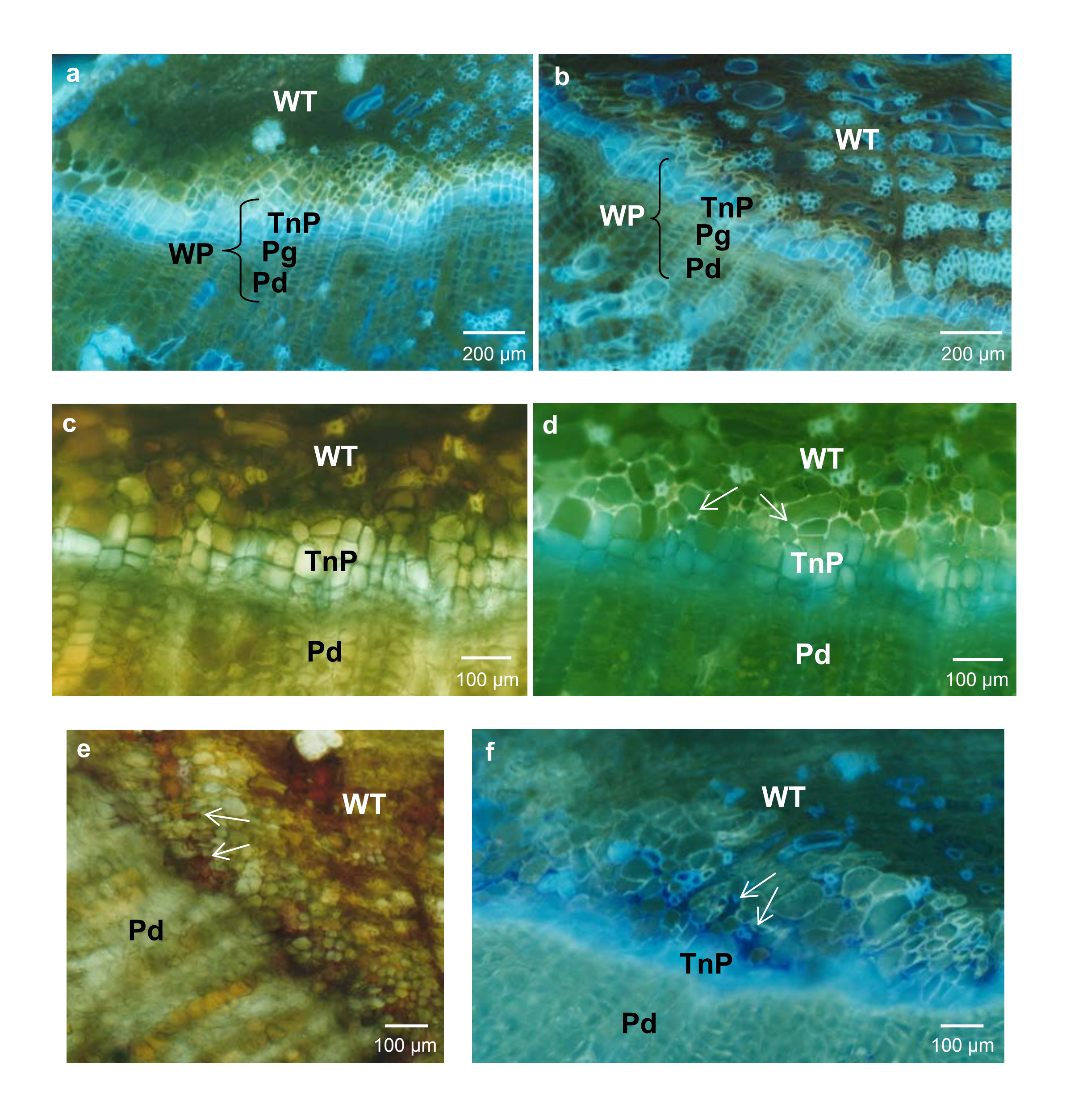


Figure 5.10: Periderm formation in *Eucalyptus marginata* 7 days after wounding.
a+b: Wound periderm (WP) between healthy and wounded tissue (WT) in the susceptible (**a**) and resistant (**b**) genotypes. The periderm consists of the autofluorescent thin-walled phellem (TnP), the phellogen (Pg) and phelloderm (Pd).
c+d: Cell walls of the TnP in the WP are black in light microscopy (**c**) and have quenched autofluorescence (**d**) when stained for suberin with Sudan Black B. Fluorescence is not quenched for lignin external to the TnP, which stains positively with Phloroglucinol + HCl (PHCl) (white arrows).
e+f: Intercellular spaces in cells adjacent to the WP react positively to PHCl, staining red in light microscopy (**e**) and having quenched autofluorescence (**f**) (white arrows).

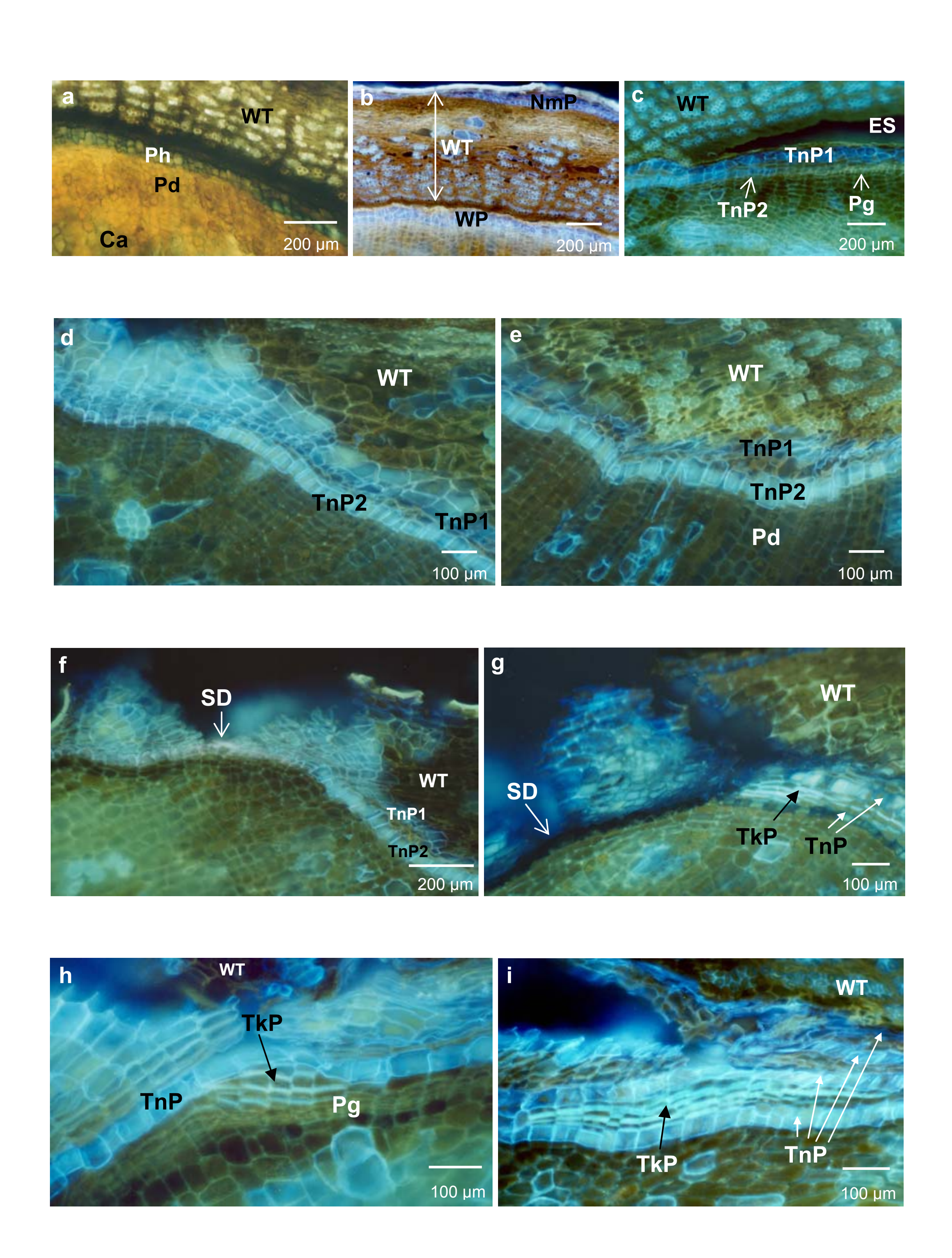

a
WT
Ph
Pd
Ca
200 µm
b
NmP
WT
WP
200 µm
c
WT
ES
TnP1
TnP2
Pg
200 µm
d
WT
TnP2
TnP1
100 µm
e
WT
TnP1
TnP2
Pd
100 µm
f
SD
WT
TnP1
TnP2
200 µm
g
WT
SD
TkP
TnP
100 µm
h
WT
TkP
TnP
Pg
100 µm
i
WT
TkP
TnP
100 µm

Figure 5.11: Periderm formation in clonal *Eucalyptus marginata* at 14-42 days after wounding (AW).

a+b: A wound periderm (WP) formed between the callus (Ca) and wounded tissue (WT) at 14 days AW. The periderm consists of phellem (Ph - staining positively with Sudan Black B in **a** and autofluorescing in **b**), a phellogen (not clearly visible in these sections) and phelloderm (Pd).

c: Two layers of TnP, with the second phellem layer (TnP2) petering out into a phellogen (Pg).

d+e: An orderly layer of TnP with cubical cells (TnP2) at 21 days AW, surrounded externally by a distorted phellem layer (TnP1) (**d+e**) and other autofluorescent tissue at the breaking edge of the wound (**d**).

f+g: Pale-pink autofluorescent material at the breaking edge of the wound at 21 (**f**) and 28 (**g**) days AW, which stains positively for suberin (**g**). Thick-walled phellem (TkP) is autofluorescing between two layers of quenched TnP in the section stained with Sudan Black B (**g**).

h: TkP surrounded internally by a phellogen at 28 days AW, with no indication of a suberised, TnP layer between the two.

i: Multiple layers of TnP at the breaking edge of the wound at 42 days AW, with TkP sandwiched between the TnP layers.

Further abbreviations (for anything not previously mentioned in text): ES = empty space, NmP = normal periderm.

5.3.3.2 Experiment 2: Colonisation by *P. cinnamomi* trial

Tissue damage

Tissues external to the wood were necrotic in all plants inoculated with *P. cinnamomi*, with necrosis occasionally extending into the xylem. At least 50% of the sections examined from SS plants treated with 0 g phosphite/L were girdled. No girdling was apparent in the sections examined from the other treatments. No hyphae were seen in any tissue.

Bark responses to *P. cinnamomi* up to 6 days after phosphite treatment

The earliest response visible with histochemical testing was the deposition of lignin at the lesion edge in cell walls of the cambial zone and phloem parenchyma cells (Figures 5.12a+b). Occasionally, these cell walls also stained positively for suberin. The staining of recent cambial derivatives was from two to more than 20 cells wide. Over time, lignin and suberin were deposited in cell walls through the phloem and cortex (Figures 5.12c-h). The majority of staining occurred in cells that existed prior to the time of injury. In some samples, meristematic activity in the plane parallel to the lesion edge was evident in healthy tissue adjacent to the stained cells (Figures 5.12g, i+j). This activity heralded the formation of a wound periderm.

The timing of the lignification and suberisation described above depended on the phosphite treatment and genotype. In untreated plants of the susceptible clonal line (SS/0), the deposition of lignin was limited to 50% or less of the ramets and no suberin was detected (Figure 5.13). In the RR/0 treatment, at least 75% of the ramets had lignin deposits. Suberin was detected in the RR/0 treatment, but its deposition was more limited than that observed for lignin in RR/0.

At 4 and 6 days after spraying, phosphite significantly increased the percentage of SS plants that laid down lignin and suberin, and the percentage of RR plants that laid down suberin (Figure 5.13). The chemical had no significant effect

on lignin deposition in RR plants (Figure 5.13). There was no genotypic difference in the deposition of lignin in the 2.5, 5 or 10 g phosphite/L treatments. At least 83% of the ramets receiving phosphite treatment had lignin deposited in the cambial zone and phloem at each harvest. A genotypic difference was observed in the timing of suberin deposition in ramets treated with 2.5, 5 or 10 g phosphite/L. In comparison with the resistant genotype, significantly fewer plants of the susceptible clonal line had produced suberin at 2 days after treatment, but there was no significant genotypic difference by 4 days after treatment (Figure 5.13).

Bark responses to *P. cinnamomi* at 24 days after phosphite treatment

A continuous periderm separated apparently healthy tissue from lesioned tissue in all samples from plants that remained alive at 24 days after phosphite treatment (Figures 5.14a-e). The periderm extended from the cambial zone and terminated at the cuticle, occasionally taking a detour to encompass a lesion patch (Figure 5.14f) or phloem fibre. Other phloem fibres near the junction of lesioned and healthy tissue were completely surrounded by a whorl of periderm (Figure 5.14d). Some of the phellem layers consisted of cubical TnP, while other were less orderly. TkP was observed in some samples between two TnP layers.

Lesioned tissue was in the process of being sloughed off in all samples, except in the RR/0 treatment (Figure 5.15b). The production of callus in adjacent healthy tissue near the xylem pushed the lesioned tissue away from the xylem (Figure 5.15c+d). Cracks appeared on the outer edge of plants in which there was callus formation and sloughing (Figure 5.15e+f). The most extensive callus formation occurred in ramets in the SS/10, RR/5 (Figure 5.14c) and RR/10 treatments. A red pigmentation was observed in the callus in unstained sections, suggesting the presence of polyphenolics. The VC was regenerating in the callus as a continuation from the neighbouring undamaged VC (Figure 5.14c). No callus

formation or regeneration of VC was observed in the RR/0 treatment (Figures 5.14a and 5.15b).

Xylem responses to *P. cinnamomi*

Occasionally, the lignification and suberisation observed in the bark response to *P. cinnamomi* extended into the xylem in cells existing before the injury. This was generally in radial alignment with the bark response and restricted to the outermost cell layers.

In areas of the stem where the VC was healthy, two to eight cell layers of traumatic parenchyma were formed instead of xylem (Figures 5.16b-d). This occurred in all treatments except SS/0. The cell walls of the parenchyma were unlignified at 2, 4 and 6 days after phosphite treatment, but were mostly lignified by 24 days after treatment (except in the SS/10 treatment, where they remained unlignified). Xylem vessels were either absent or greatly reduced in diameter in the traumatic tissue. Frequently, the parenchyma cells were filled with polyphenolics (Figures 5.16c+d). By 4 days after phosphite treatment, it was evident that the VC had returned to normal xylem production, with a few layers of lignified xylem cells (including vessels) produced since the formation of traumatic parenchyma.

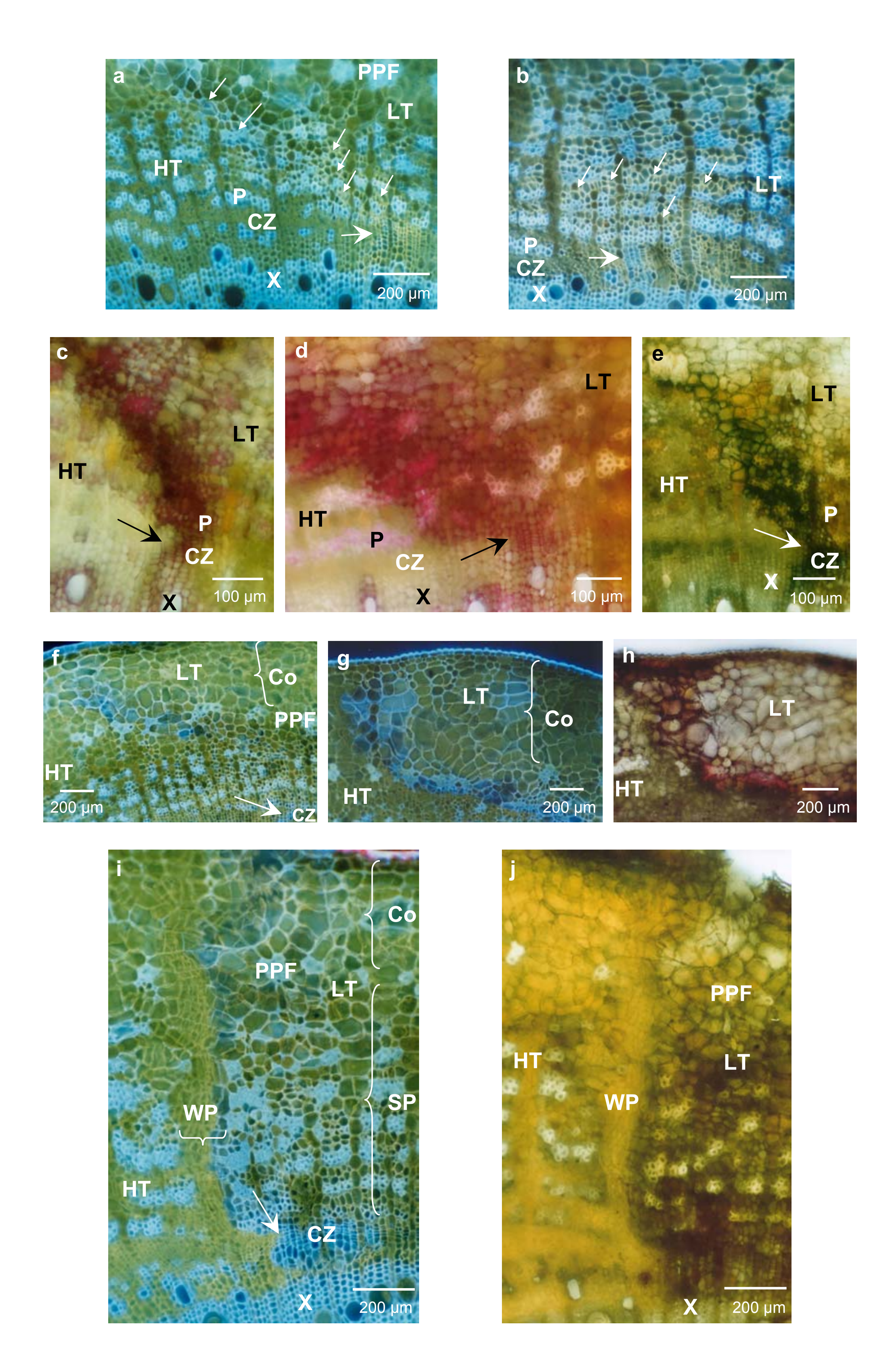

a
PPF
LT
HT
P
CZ
X
200 µm
b
LT
P
CZ
X
200 µm
c
LT
HT
P
CZ
X
100 µm
d
LT
HT
P
CZ
X
100 µm
e
LT
HT
P
CZ
X
100 µm
f
LT
Co
PPF
HT
200 µm
CZ
g
LT
Co
HT
200 µm
h
LT
HT
200 µm
i
Co
PPF
LT
SP
WP
HT
CZ
X
200 µm
j
PPF
HT
LT
WP
X
200 µm

Figure 5.12: Response to *Phytophthora cinnamomi* at the junction of healthy tissue (HT) and lesioned tissue (LT) in the cambial zone (CZ), phloem (P) and cortex (Co) in stems of *Eucalyptus marginata* treated with phosphite. Clonal lines classified as resistant (RR) and susceptible (SS) to *P. cinnamomi* were sprayed with 2.5, 5 or 10 g phosphite/L at 4 days after inoculation.

a+b: Increased autofluorescence in the CZ (large arrowheads) and P (small arrowheads) in the SS/10 (**a**) and RR/10 (**b**) treatments at 4 days after spraying.
c-e: Lignin (**c+d**) and suberin (**e**) deposition in walls of cells in the CZ (large arrowheads) and P in the SS/10 (**c**), SS/5 (**d**) and SS/2.5 (**e**) treatments at 6 days after spraying. Sections were stained with Phloroglucinol + HCl (PHCl - **c+d**) and Sudan Black B (**e**), and examined under brightfield light.
f: Increased autofluorescence in CZ (large arrowhead) and in the P and Co tissues in the RR/2.5 treatment at 6 days after spraying.
g: Increased autofluorescence in the Co and P in the SS/2.5 treatment at 6 days after spraying. There is some meristematic activity internal to the autofluorescent cells in the phloem. The fluorescent cells external to and in radial alignment with the meristematic layer (phellogen) are newly differentiated phellem of a wound periderm (WP). Cells existing prior to injury are also autofluorescent.
h: The same plant as **g**, showing lignin deposition in cell walls in a section stained with PHCl and examined under brightfield light.
i: Developing WP in an RR plant treated with 5 g phosphite/L at 6 days after spraying. The WP is adjacent to phloem fibres and other fluorescent cells existing prior to injury. Cells in the CZ at the junction of healthy and lesioned tissue have autofluorescent walls (large arrowhead).
j: The same plant as **i** viewed under brightfield light, showing WP separating HT from LT.
Further abbreviations (for those not mentioned previously in text): PPF = primary phloem fibre, SP = secondary phloem and X = xylem.

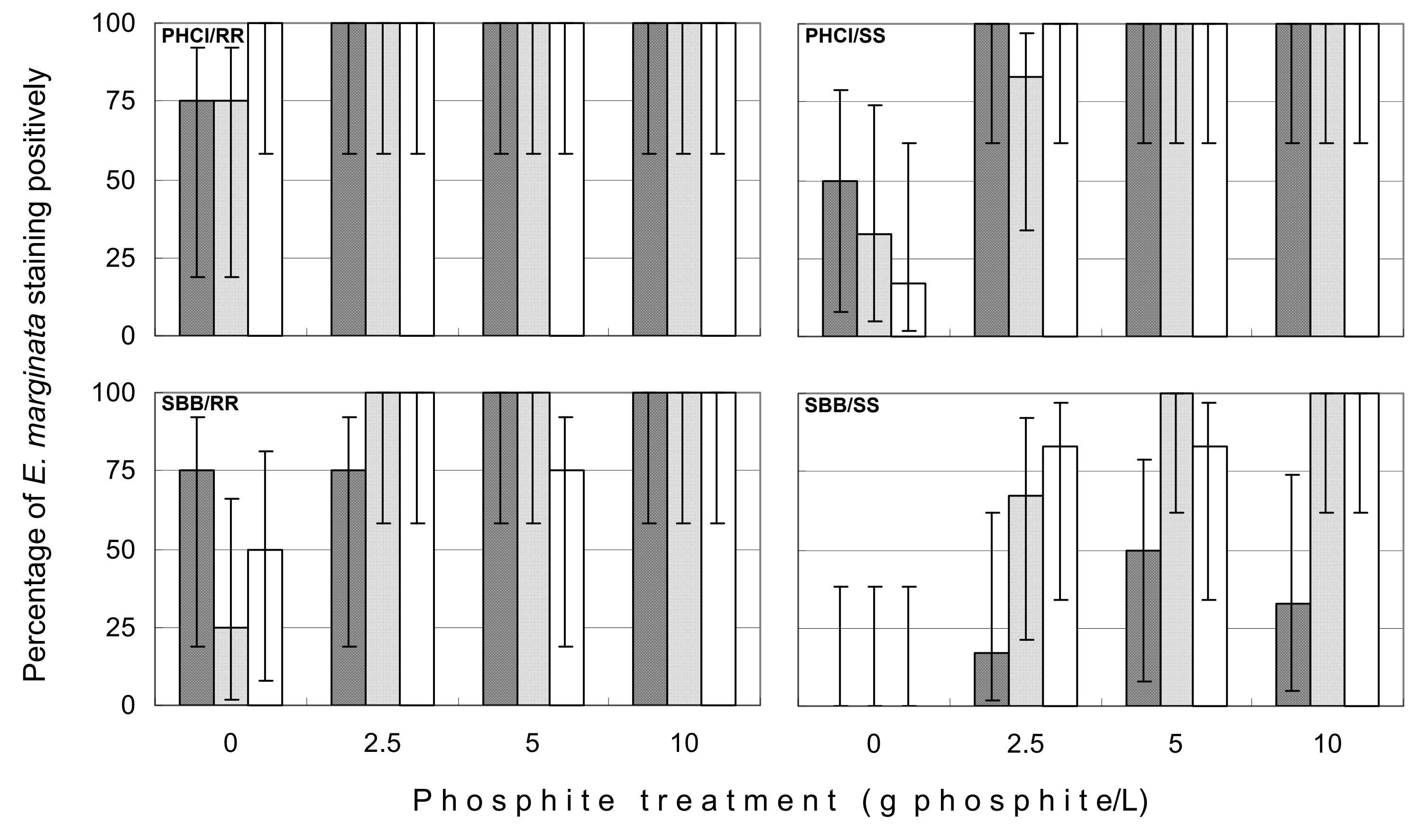


Figure 5.13: Percentage of *Eucalyptus marginata* ramets [resistant (RR) and susceptible (SS) clonal lines] observed staining positively with Phloroglucinol + HCl (PHCl – indicative of lignin) and Sudan Black B (SBB – indicative of suberin) in bark tissue ± 95% confidence intervals (n=4 for RR and n=6 for SS). Leaves were sprayed with phosphite 4 days after the stem was inoculated with *Phytophthora cinnamomi*, and the plants were harvested at 2 (▩), 4 (▨) and 6 (□) days after phosphite treatment.

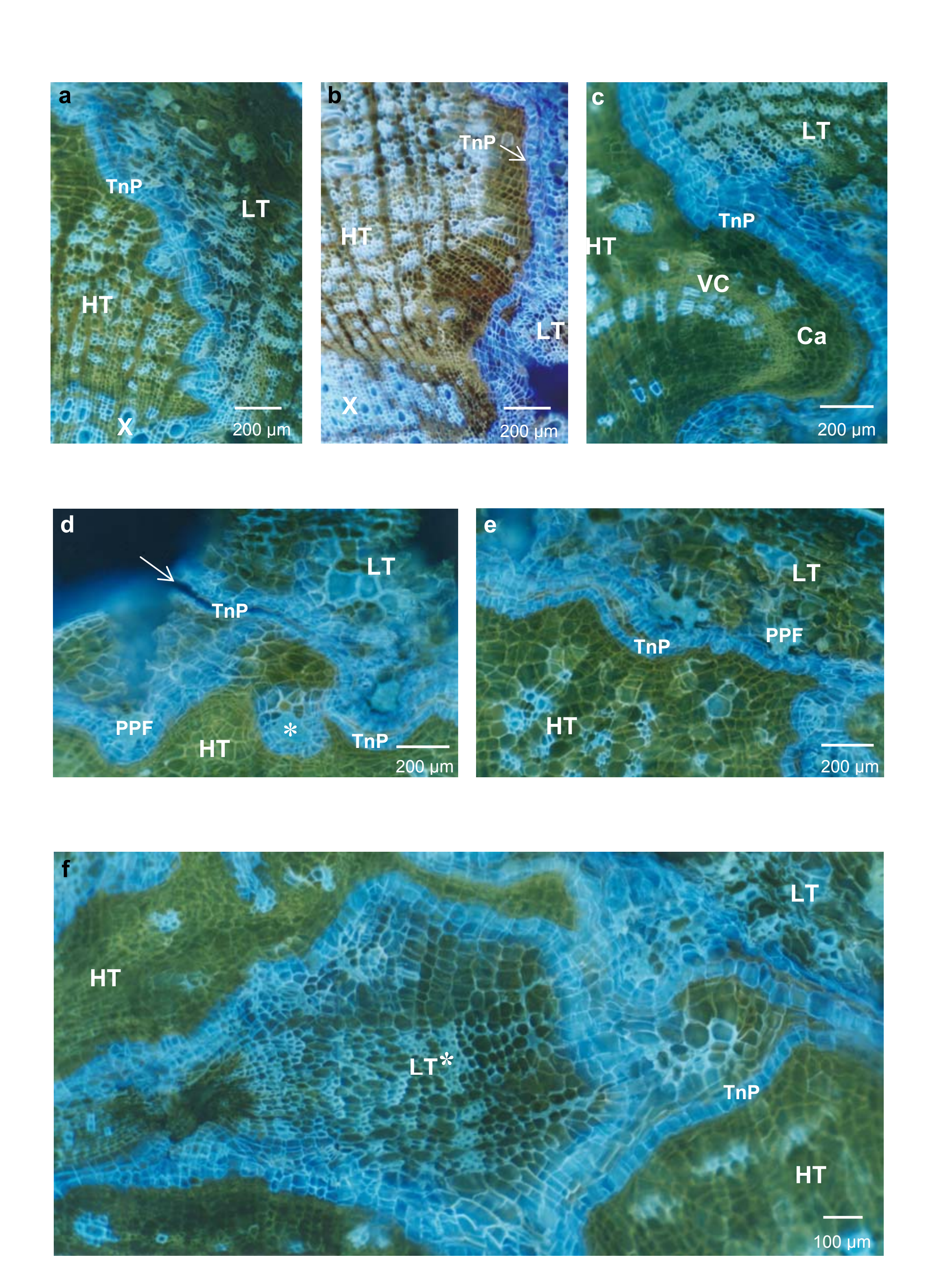
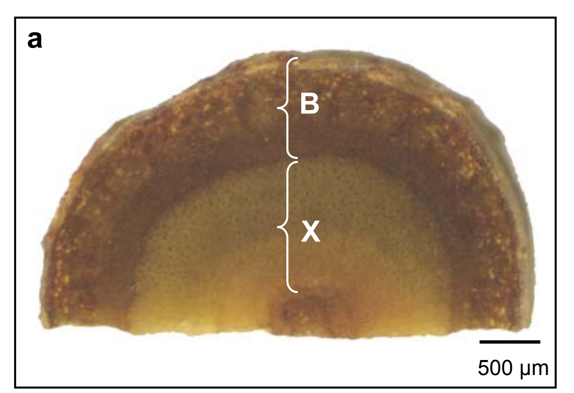

a
TnP
LT
HT
X
200 µm
b
TnP
HT
LT
X
200 µm
c
LT
TnP
HT
VC
Ca
200 µm
d
LT
TnP
PPF
HT
*
TnP
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e
LT
PPF
TnP
HT
200 µm
f
LT
HT
LT*
TnP
HT
100 µm

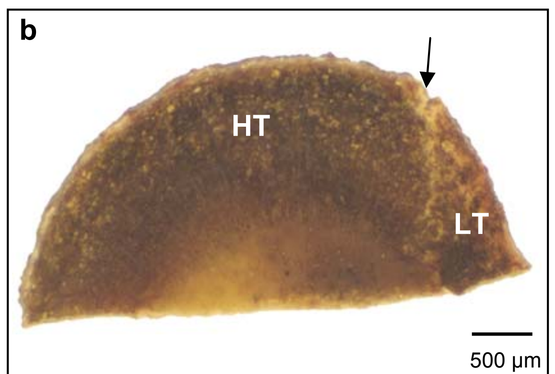
Figure 5.14: Response to *Phytophthora cinnamomi* at the junction of healthy tissue (HT) and lesioned tissue (LT) in stems of *Eucalyptus marginata* at 24 days after spraying with phosphite. Clonal lines classified as resistant (RR) and susceptible (SS) to *P. cinnamomi* were sprayed with 0, 2.5, or 5 g phosphite/L at 4 days after inoculation.

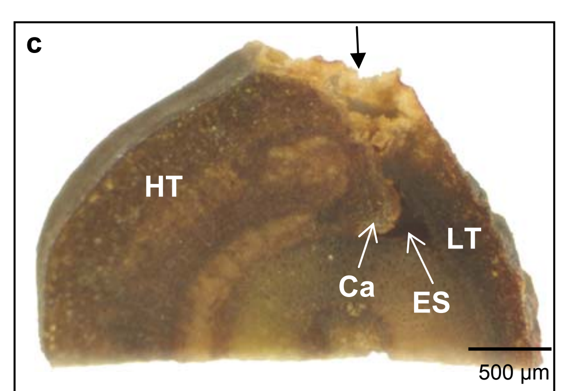
a-c: A continuous periderm formed between healthy and lesioned tissue through the cambial zone and phloem tissue in the RR/0 (**a**), RR/2.5 (**b**) and RR/5 (**c**) treatments. The thin-walled phellem (TnP) cells of the periderm are autofluorescent. Extensive callus (Ca) production is visible in **c**. Within the callus, a new vascular cambium (VC) is forming.

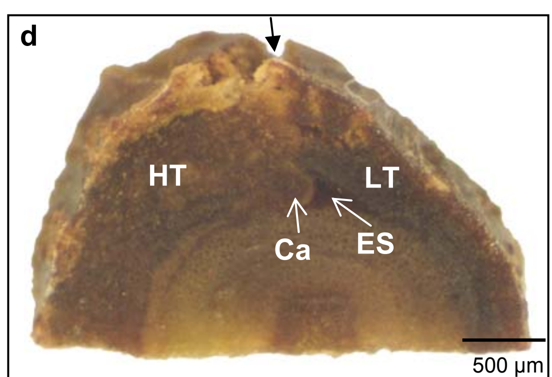
d-f: Periderms with autofluorescent TnP cells separating healthy tissue from lesioned tissue in the SS/2.5 treatment. In **d**, primary phloem fibres (PPF) are surrounded by a whorl of TnP cells (indicated by an asterisk). The lesioned cortical tissue is starting to break away from the healthy tissue (indicated by an arrowhead), with a layer of TnP formed on the outer surface of the remaining healthy tissue. In **f**, a lesion patch (indicated by an asterisk) is completely surrounded by TnP cells.

Further abbreviations (for those not previously mentioned in text): X = xylem.









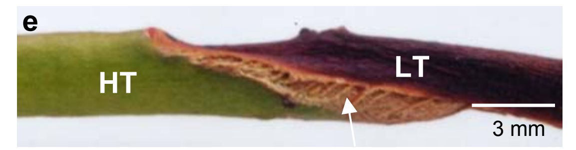




Figure 5.15: Macroscopic responses to *Phytophthora cinnamomi* at the junction of healthy tissue (HT) and lesioned tissue (LT) in stems of *Eucalyptus marginata* at 24 days after spraying with phosphite. Clonal lines classified as resistant (RR) and susceptible (SS) to *P. cinnamomi* were sprayed with 0, 5 or 10 g phosphite/L at 4 days after inoculation.

a: Healthy stem in the SS clonal line, showing location of bark (B) and xylem (X).

b: Absence of callus production in the RR/0 treatment.

c+d: Callus (Ca) production lifting the lesioned tissue away from the xylem in the RR/5 (**c**) SS/10 (**d**) treatments, leaving an empty space (ES) and resulting in cracking at the bark surface (indicated by arrowheads).

e+f: External view of cracking (indicated by arrowheads) in the RR/5 (**e**) and SS/10 (**f**) treatments at the junction of healthy and lesioned tissue.

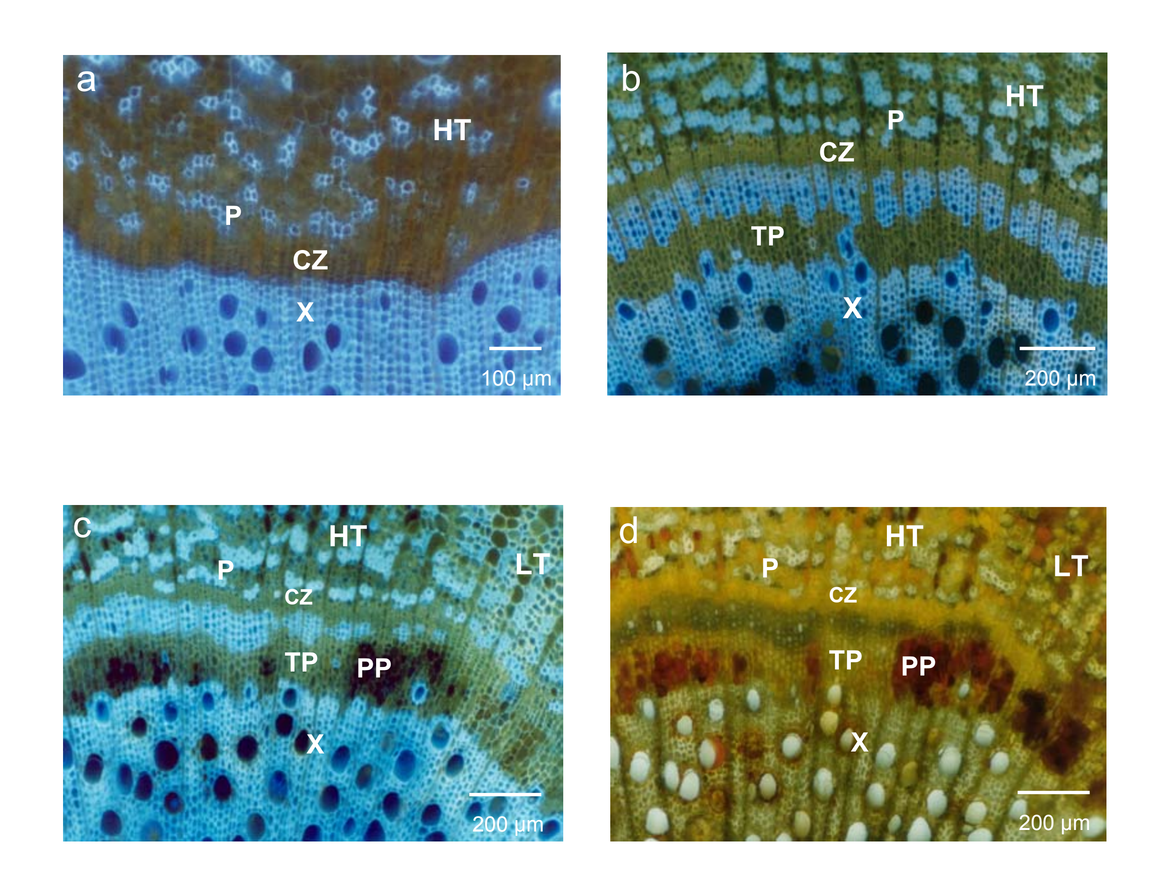


Figure 5.16: Response to *Phytophthora cinnamomi* in the xylem of *Eucalyptus marginata* treated with phosphite, adjacent to healthy phloem tissue. The clonal line classified as susceptible (SS) to *P. cinnamomi* was sprayed with 0, 2.5 or 5 g phosphite/L at 4 days after inoculation.

a: Cambial zone (CZ) in uninoculated healthy plant, surrounded externally by phloem tissue (P) and internally by xylem tissue (X).

b: Formation of unlignified traumatic parenchyma (TP) by the vascular cambium at 6 days after spraying with 2.5 g phosphite/L. Xylem vessels are greatly reduced in diameter.

c+d: Polyphenolic (PP) deposits in TP cells produced by the vascular cambium in healthy tissue (HT) adjacent to lesioned tissue (LT) at 4 days after spraying with 5 g phosphite/L.

5.4 Discussion

Post-inoculation treatment with phosphite effectively reduced the lesion extension in both susceptible and resistant clones of *E. marginata* inoculated with *P. cinnamomi*. This contrasts with the results of Marks and Smith (1992), where the treatment of *Leucodendron* hybrids with phosphite at 10 days after inoculation did not restrict the length of lesions caused by *P. cinnamomi*. This is most likely a result of the higher applied concentrations in the current study (2.5 g phosphite/L and higher compared with 1 g phosphite/L). A range of other factors may have also contributed to the difference in control, including the timing of phosphite application, phosphite uptake and distribution, isolate pathogenicity, isolate sensitivity to phosphite, host susceptibility and environmental conditions.

Unlike lesion extension, phosphite treatment had little effect on the mortality of infected plants in the susceptible clonal line of *E. marginata*, except in the SS/10 treatment. Death was generally delayed but not prevented in the treated SS plants. It is likely that several of the plants had already sustained irreversible damage at the time of phosphite treatment, as discussed in Chapter 4. The extent of circumferential spread by *P. cinnamomi* was not measured at the time of treatment in the current study, but death was always associated with stem girdling. However, an experiment that tested the effect of girdling on the survival of clonal *E. marginata* in the absence of *P. cinnamomi* showed that plants remained alive for at least one month after girdling (data not presented). McCredie *et al*. (1985) suggested that infected *Banksia* plants die as a result of stem girdling. Other researchers have proposed that toxins (Woodward *et al*., 1980) or a hormonal imbalance (Cahill *et al*., 1986) are responsible for the death of infected eucalypt seedlings. The cause of death in plants infected with *P. cinnamomi* is still not clear (Shearer and Smith, 2000). Whatever the cause, results from the current study suggest that clones of *E. marginata* are able to survive infection by *P. cinnamomi* when the circumferential

spread is restricted.

In the current study, lesion lengths were not a good indication of the extent of stem colonisation by *P. cinnamomi* in the resistant clonal line, where the pathogen was found to extend further beyond the lesion than in the susceptible clonal line. Extension of *P. cinnamomi* beyond the lesion was noted previously in Chapter 3, and as discussed in Chapter 3, has been observed by a number of other researchers (Davison *et al*., 1994; O'Gara *et al*., 1997; Hüberli *et al*., 2000). Damage caused by *P. cinnamomi* was noted to extend furthest in the cortex and outer phloem in transverse sections in both genotypes, which does not suggest that internal lesions were present and overlooked. This finding differs from the histological studies of Bucciarelli *et al*. (1999), where tissues primarily colonised by the pathogen varied according to the host genotype in the interaction between *Populus tremuloides* and *Entoleuca mammata*. Bucciarelli *et al*. (1999) reported that the leading edge of penetration by the pathogen was in cortical and phloem cells in the susceptible genotype, but was along the vascular cambium in the resistant genotype. Unfortunately, the hyphae of *P. cinnamomi* were difficult to distinguish in sections of *E. marginata*, unlike the hyphal aggregates of *En. mammata* shown in Bucciarelli *et al*. (1999). The current study relied upon visible damage rather than the exact location of the pathogen, and any areas of minimal damage could easily have remained undetected with the thick hand sections. Hand sectioning did, however, provide a quick method for conducting the histological studies.

Microscopic examination of stems with restricted lesion extension revealed histochemical and anatomical changes at the lesion front similar to those described as non-specific responses of bark to injury (Mullick, 1977; Biggs *et al*., 1984; Biggs, 1992). A boundary zone and wound periderm formed between healthy and damaged bark tissue. However, in contrast to Mullick's description of the boundary

zone as non-suberised (Mullick, 1975), the boundary zone in *E. marginata* was usually a ligno-suberised layer, as previously reported by Tippett and Hill (1984) in *E. marginata* roots. Exceptions to this occurred mostly in the SS/0 treatment, in which suberin was notably absent from infected stems. The production of lignin was clearly not enough to inhibit *P. cinnamomi*. Although lignin has been proposed to play an important role in disease resistance (Vance *et al*., 1980), it was found to be ineffective against the invasion of chestnut bark by *Endothia parasitica* (Bramble, 1936). Cahill *et al*. (1993) observed that clones of 11J402 (SS) responded to *P. cinnamomi* with an increase in lignin but were still susceptible to lesion development. In a study by Casares *et al*. (1986), *P. cinnamomi* was found to have the capacity to degrade lignin. Suberin is reported to be an effective barrier to pathogen invasion (Kolattukudy, 1984), although there are fungi known to degrade suberin, such as *Armillaria mellea* (Swift, 1965). It is not known whether *P. cinnamomi* can degrade suberin, although the pathogen has been reported to 'break out' from arrested lesions (Tippett *et al*., 1983), 'break through' wound periderm (Marks and Smith, 1992) and infect non-wounded periderm tissue (O'Gara *et al*., 1997). It has been suggested that the success of suberised layers in protecting peach bark from *Leucostoma* spp. is related to the rate and amount of suberin accumulation (Biggs and Miles, 1988; Biggs, 1989). This may also determine the resistance of *E. marginata* to *P. cinnamomi*. Unfortunately, the current study did not examine the amount of suberin accumulation in *E. marginata*.

The production of traumatic parenchyma to form a barrier zone in xylem has been observed in several plant genera in response to abiotic wounding or infection, including *Acer* (Mulhern *et al*., 1979; Rademacher *et al*., 1984; Pearce and Woodward, 1986), and *Eucalyptus* (Skene, 1965; Chudnoff, 1971; Tippett, 1986; Wilkes, 1986). In *Eucalyptus* spp., the production of a band of traumatic parenchyma is an early stage of kino vein formation (Tippett, 1986). Chapter 4

showed that *E. marginata* responded to *P. cinnamomi* with the production of kino veins, but also concluded that kino was not likely to be effective at protecting *E. marginata* from the pathogen. Consequently, the barrier zone was not examined in detail.

Although the effect of phosphite on lesion extension was evident by 2 days after the foliar treatment of *E. marginata* clones, the indirect mode of phosphite action was generally not observed microscopically until 4 days after treatment. Defence responses were stimulated at all phosphite concentrations in the current study. This contrasts with the work of Afek and Sztejnberg (1989) and Jackson *et al*. (2000), where there was no indication of an indirect mode of action at high concentrations of phosphite. Pre-inoculation treatments of citrus (Afek and Sztejnberg, 1989) and *E. marginata* (Jackson *et al*., 2000) were presumed to inhibit the pathogen directly before it had a chance to damage the host. In the present study, post-inoculation treatment with phosphite was sufficient to restrict the extension of *P. cinnamomi*, but not to kill the pathogen. Thus there is the potential for *P. cinnamomi* to 'break out' when the levels of phosphite decline, particularly if the periderms can be circumvented.

Phosphite stimulated the defence responses in both clonal lines, which suggests that the resistant genotype was only moderately resistant. Nemestothy and Guest (1990) reported no additive effect of Fosetyl-Al on the resistance of resistant tobacco cultivars to *P. nicotianae*, while Guest and Bompeix (1990) suggested that phosphite could enhance the resistance of moderately resistant plants. Previous studies on citrus (Khan *et al*., 1986; Afek and Sztejnberg, 1989) and tobacco (Guest, 1984; Guest *et al*., 1989) found that defence responses of susceptible cultivars after treatment with Fosetyl-Al or phosphite resembled those of untreated naturally resistant cultivars. In *E. marginata*, phosphite treatment of clones in the susceptible genotype resulted in stronger defence responses than that

observed in the RR/0 treatment, particularly at 24 days after treatment. Temperatures in the glasshouse were relatively high during the current study, and high temperatures have been shown to reduce the resistance of clonal *E. marginata* to *P. cinnamomi* (Hüberli *et al*., 2002). The combined effect of temperature and phosphite on the resistance of clonal *E. marginata* is investigated in the following chapter.

Clones of the susceptible genotype clearly possessed the machinery required to respond to injury. This was evident not only in the response of infected plants treated with phosphite, but also in the response to abiotic wounding. Enebak *et al*. (1997) noted a similar pattern in two genotypes of aspen, in which the resistant and susceptible genotypes both compartmentalised abiotic wounds but the susceptible clones were not able to respond to infection by the pathogen. Although wound compartmentalisation and/or closure in trees is suggested to be under genetic control (Shigo *et al*., 1977; Garrett *et al*., 1979; Armstrong *et al*., 1981; Shigo, 1989), no difference was evident in the wound response of the two clonal lines of *E. marginata*.

The observed formation of cubical phellem cells internal to more variable-shaped phellem cells following wounding has been described previously by Tippett and Hill (1984). Tippett and Hill (1984) considered the cubical phellem of *E. marginata* to be part of an exophylactic or normal periderm internally abutting the necrophylactic (wound) periderm. Current confusion as to what distinguishes an exophylactic periderm from a necrophylactic periderm (outlined in Chapter 1) makes it unwise to adopt this terminology. In the current study, it was not possible to determine whether the cubical phellem originated from the phellogen of the wound periderm or a new phellogen formed internally. The origin of TkP cells is similarly difficult to determine, and has been discussed in the literature. Chattaway (1953), Grozdits *et al*. (1982), Godkin *et al*. (1983) and Robinson (1997) consider that the

phellogen of the wound periderm forms alternate layers of TnP and TkP. In contrast, Chang (1954) and O'Gara (1998) suggest that TkP is formed by the lignification of redundant phellogen and phelloderm after a new phellogen forms internally. As pointed out in Srivastava (1964), this is only feasible if the new phellogen arises in the phelloderm produced by the previous periderm, given the radial alignment of the cells. It was not possible in the current study to identify if either or both of these two scenarios occur in *E. marginata*.

Wounding has been described as a "potent initiator of mitotic activity" (Lipetz, 1970), and this was evident in the majority of abiotically wounded plants. Although not statistically significant, the occurrence of two plants with reduced callus formation after treatment with phosphite suggests that the chemical may have disrupted the wound response. No such effect was evident in the presence of *P. cinnamomi*, where phosphite stimulated mitosis as part of the defence response, with meristematic activity involved in the compartmentalisation of damaged tissue (formation of periderms) and closure of healthy tissue (callus). Phosphite has been found to cause mitotic abnormalities in root tips of broad beans and petunias (Fairbanks *et al*., 2002), and growth abnormalities have been observed in Proteaceous and Myrtaceous species at operational spray concentrations (Barrett and Grant, 1998). Phytotoxicity was the only detrimental effect of phosphite observed in healthy tissues of *E. marginata* in the current study, with 10 g phosphite/L unacceptably phytotoxic to the resistant genotype. Interestingly, this treatment did not provide the RR clones with as much protection from *P. cinnamomi* as the 2.5 or 5 g phosphite/L treatments, yet provided the SS clones with the best protection from lesion extension and plant mortality.

Chapter 6: Influence of temperature and phosphite treatment on the responses of clonal *Eucalyptus marginata* to abiotic wounding and colonisation by *Phytophthora cinnamomi*

6.1 Introduction

In Chapter 5, phosphite restricted lesion extension by *P. cinnamomi* in stems of *E. marginata*, but had little effect on the mortality of inoculated plants. Extensive establishment of the pathogen in the host before phosphite treatment and high temperatures in the glasshouse may have limited the effectiveness of phosphite. Swart and Denman (2000) also proposed that temperature influenced the effectiveness of phosphite in the containment of *P. cinnamomi* in *Leucospermum* hybrids. The effects of temperature on the efficacy of phosphite *in vivo* have not been tested previously.

Temperature affects host-*P. cinnamomi* interactions, with an increase in the severity of disease as temperatures increase up to 30°C (Zentmyer, 1981; Grant and Byrt, 1984; Shearer *et al*., 1987). Erwin and Ribeiro (1996) comment that even plants with general resistance to *Phytophthora* are susceptible if environmental conditions are optimum for the pathogen. In a recent study of the *E. marginata*-*P. cinnamomi* interaction, the level of resistance in clonal lines changed with temperature (Hüberli *et al*., 2002). Clonal line 1J30 was resistant to *P. cinnamomi* at 15 and 20°C but susceptible at 25 and 30°C after underbark inoculation with an isolate showing optimum *in vitro* growth at approximately 28°C (Hüberli, 1995).

Tippett *et al*. (1983) described the interaction between *E. marginata* and *P. cinnamomi* as a rates game, with the outcome being dependent upon the growth rates of the pathogen and the speed with which the host can respond. Temperature influences the rate of wound responses in peach trees (Biggs and Northover, 1985;

Biggs, 1986), almond trees (Doster and Bostock, 1988) and carrots (Garrod *et al*., 1982). Wound responses such as the formation of a ligno-suberised boundary layer and wound periderm were found to occur most rapidly at the highest temperatures tested in each of these studies.

The aims of this chapter were to examine the effects of temperature and phosphite treatment on 1) the outcome of the *E. marginata*-*P. cinnamomi* interaction, including the extent of colonisation by the pathogen and the histological responses of clonal *E. marginata* to *P. cinnamomi*, and 2) the histological responses of *E. marginata* to shallow abiotic wounding.

6.2 Materials and methods

Three experiments were conducted to achieve the aims of the present study. The first experiment focused on the histological responses of *E. marginata* to abiotic wounding and phosphite treatment at two temperatures, with a preliminary look at the effect of the two temperatures on colonisation by *P. cinnamomi*. The second experiment examined the effect of phosphite treatment on disease development and histological responses of *E. marginata* after inoculation with *P. cinnamomi* at the two temperatures used in Experiment 1. The third experiment was a preliminary look at the effect of two temperatures intermediate to those used in Experiments 1 and 2 on disease development and histological responses of *E. marginata* inoculated with *P. cinnamomi*.

6.2.1 Experimental design

6.2.1.1 Experiment 1

The independent variables for the wounded plants were temperature (20 and 28°C), clonal line of *E. marginata* (SS and RR), and treatment of ramets with 0 and 5 g phosphite/L immediately after wounding the stem with a razor blade. The

dependent variables were the change in stem diameter and the histological response of the stems to wounding at 7 days post-wounding.

The independent variables for the inoculated plants were temperature (20 and 28°C) and clonal line of *E. marginata* (SS and RR), while the dependent variables were the daily acropetal and circumferential lesion extension, the extent of colonisation by *P. cinnamomi* beyond the lesion and the incidence of girdling and wilting at 10 days after inoculation with *P. cinnamomi*. For each temperature, the plants were arranged in randomised complete blocks. There were six replicate plants for each treatment.

6.2.1.2 Experiment 2

The independent variables were temperature (20 and 28°C), clonal line of *E. marginata* (SS and RR) and treatment with 0 and 5 g phosphite/L at 2 days after inoculation with *P. cinnamomi*. The dependent variables were the daily lesion extension (acropetal and circumferential), extent of colonisation by *P. cinnamomi* beyond the lesion, the incidence of girdling and wilting, and the histological responses of the stems at 7 days after phosphite treatment. For each temperature, the plants were arranged in randomised complete blocks. There were eight replicate plants for each treatment, plus four SS ramets for each temperature as wounded and uninoculated controls. The experiment was conducted twice over a 2-month period, with the 20°C cabinet changed to 28°C and vice versa for the other cabinet in the repeat experiment.

6.2.1.3 Experiment 3

The independent variables were temperature (23 and 24°C) and clonal line of *E. marginata* (SS and RR). The dependent variables were the daily lesion extension (acropetal and circumferential), extent of colonisation by *P. cinnamomi* beyond the

lesion, and histological response of the stems to colonisation by *P. cinnamomi* at 9 days post-inoculation. There were five replicate plants for each treatment.

6.2.2 Plant material

The Marrinup Nursery (Alcoa World Alumina Australia) provided ramets of two clonal lines of *E. marginata* (SS and RR - refer to Section 5.2.2 for genet details). In June 1999, the 9-month-old ramets were planted into 130 mm free-draining pots. The potting mix was the same as that described in Section 5.2.2, and was top-dressed with the slow-release fertiliser in September 1999. Plants were watered daily by an automatic drip watering system prior to the commencement of each experiment. The height and stem diameter of the plants immediately before wounding/inoculation ranged from 40-64 cm and 3-7 mm, respectively.

6.2.3 Experimental conditions

The experiments were conducted in February 2000 (Experiment 1), March/April 2000 (Experiment 2) and May 2000 (Experiment 3). Plants were transferred to controlled environment cabinets (Environ Air, SRJ Cabinet Sales, Greenacre, Australia) 1-2 weeks prior to the commencement of each experiment, and were hand-watered daily. The cabinets were set to give a day/night cycle of 14.5/9.5 hours.

In Experiments 1 and 3, the temperatures in the cabinets were measured using a digital maximum/minimum thermometer (Rowe Scientific, Australia), while in Experiment 2, Tinytag Plus Data Loggers (Gemini Data Loggers Ltd, United Kingdom) measured the temperature at hourly intervals. The mean temperatures recorded were always within 0.5°C of the intended temperature (Table 6.1).

Table 6.1: Mean temperatures and range of temperatures recorded in growth cabinets during Experiments 1-3. *This cabinet's circulation system malfunctioned during the experiment, but was repaired within 24 hours.

Experiment (intended temperature - °C)	Mean temperature (±SE) (°C)	Range (°C)
1 (20)	19.9 ± 0.4	16.5 – 21.8
1 (28)	27.5 ± 0.8	*14.4 – 29.8
2a (20)	19.9 ± 0.01	18.0 – 20.8
2a (28)	27.9 ± 0.01	26.5 – 29.1
2b (20)	20.0 ± 0.01	18.3 – 20.8
2b (28)	27.8 ± 0.01	26.5 – 29.1
3 (23)	23.1 ± 0.1	22.4 – 23.7
3 (24)	24.0 ± 0.05	23.7 – 24.3

6.2.4 Inoculum material, inoculation and abiotic wounding procedure

The inoculum material and inoculation procedure for each experiment was the same as that described in Section 3.2.5, with the underbark inoculation of the main stems with Miracloth discs colonised with *P. cinnamomi* isolate MP94-48.

In Experiments 1 and 2, stems of *E. marginata* were wounded at 10 cm above the soil level using the same cutting method and wrapping as utilised for underbark inoculation (refer to Section 3.2.5).

6.2.5 Spray application

Plants were sprayed with phosphite on the day of wounding in Experiment 1 and 2 days after inoculation with *P. cinnamomi* in Experiment 2. Plants were removed from the cabinets and sprayed to run-off as described in Section 5.2.4. They were left to dry for 1 hour before being returned to the cabinets.

6.2.6 Monitoring

The acropetal extension of lesions from the inoculation point was measured daily until harvest. In Experiment 1, five categories were used to estimate the circumferential spread of lesions daily: 0-90, 90-180, 180-270, 270-324 and 324-360 degrees. In Experiments 2 and 3, the angle of circumferential spread was recorded daily. Plant health was also monitored daily. Stem diameters were measured at 2 mm below the wound at the times of wounding and harvest.

6.2.7 Harvest

Wounded uninoculated plants were harvested at 7 days after wounding. Wounded and healthy stem segments were cut and fixed as described in Section 5.2.7, except that the vials were not put under vacuum. In Experiment 2, a 1 cm segment above the wounded region was plated onto NARPH as described in Section 5.2.7.

In Experiment 1, plants inoculated with *P. cinnamomi* were harvested at 10 days after inoculation. A 1 cm segment of stem incorporating the highest point of the lesion edge was sliced in half longitudinally and plated onto NARPH. A 1 cm segment further into the lesion was then cut and plated, plus 1 cm segments up to 6 cm beyond the lesion to determine the extension of *P. cinnamomi* beyond the lesion.

In Experiments 2 and 3, inoculated plants were harvested at 9 days after inoculation. The following stem segments were sampled, cut in half longitudinally and plated onto NARPH: 1 cm including 7.5 mm into the lesion and 2.5 mm above the lesion; 1 cm further into the lesion (where present); 7.5 mm beyond the lesion; and a further six 1 cm segments beyond the lesion. For each plant, one half of the segment incorporating the lesion edge and the segment beyond the lesion edge were placed in fixative and not plated. The inoculation point was also placed in fixative.

Plates were incubated and examined as described in Section 3.2.6.

6.2.8 Preparation and examination of histological samples

Samples were prepared and examined as described in Section 5.2.8, with the exception that sections were not stained with PHCl in the repeat of Experiment 2 or in Experiment 3. In each run of Experiment 2, the lesion front and inoculation point were examined in five randomly selected replicate plants from each treatment, with beyond the lesion examined in four plants from each treatment.

6.2.9 Statistical analysis

Experiment 1

MANOVAs were conducted to test for the effect of temperature and genotype on acropetal and circumferential lesion spread, extension of *P. cinnamomi* beyond the lesion and total length of colonisation by *P. cinnamomi*. To compensate for pseudoreplication, treatment means were analysed, with the interaction between temperature and genotype included in the error term (Milliken and Johnson, 1989). For the circumferential spread, the midpoint of each category was used for analysis. The extension beyond the lesion and total length of colonisation were calculated as described in Section 5.2.9.

The percentages of plants that were girdled and wilted at the time of harvest were calculated, and the 95% confidence interval was used to determine if there was a significant difference between treatments. Since a MANOVA showed that neither phosphite treatment nor genotype had a significant ($P>0.05$) effect on the change in stem diameter of wounded plants, all the stem growth data was combined for each temperature. A one-way ANOVA was then conducted to test the effect of temperature on the change in stem diameter.

The percentages of plants exhibiting meristematic activity and phellem production were calculated, with the application of the 95% confidence interval to determine any significant differences between treatments.

Experiment 2

Means for the dependent variables were calculated separately for each time the experiment was run, and these means provided the raw data for analysis. Using the general linear model procedure, separate MANOVAs were conducted to test for the effect of temperature, genotype and phosphite treatment on: acropetal lesion extension, circumferential lesion spread, extension of *P. cinnamomi* beyond the lesion and total length of colonisation by *P. cinnamomi* at 9 days after inoculation with *P. cinnamomi*. Before analysis, the data were screened for assumptions of homoscedasity, normality and non-correlations of means and variances.

The numbers of plants that were girdled or wilted, and the number exhibiting meristematic activity and phellem production at the lesion edge, were calculated as a percentage of all replicates. Significant differences between treatments were determined by the use of 95% confidence intervals.

A rating system was used to estimate the extent of suberisation in healthy tissue adjacent to bark lesions, where 0 = suberin not detected, 1 = 1-50% and 2 = 51-100% cells along the lesion edge suberised (Figure 6.1). The mean ratings for each treatment were not analysed statistically.

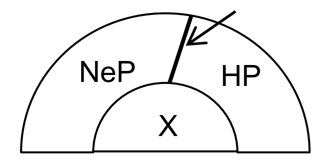


Figure 6.1: Schematic diagram of a transverse stem section showing the tissue assessed for the extent of suberisation (arrow pointing to lesion edge). Abbreviations: NeP = necrotic phloem, HP = healthy phloem and X = xylem.

Experiment 3

Using the method described for Experiment 1 to compensate for pseudoreplication, MANOVAs were conducted to test for the effect of temperature and genotype on acropetal and circumferential lesion spread, extension of *P. cinnamomi* beyond the lesion and total length of colonisation by *P. cinnamomi*.

The extent of suberisation in healthy tissue at the lesion edge was assessed using the rating system described for Experiment 2.

6.3 Results

6.3.1 Experiments 1 and 2: 20 and 28°C

6.3.1.1 Description of lesions

Discolouration of stem tissue above the inoculation point was visible by 2 days after inoculation with *P. cinnamomi*. Lesion colour ranged from pale yellow to very dark brown (Figure 6.2a+b). A white/yellow border surrounded lesions (Figure 6.2c) in 75 and 12% of plants in the RR/5 and RR/0 treatments, respectively. Histological examination of these samples determined that the 'border' resulted from cracks in the epidermis and outer cortex (Figure 6.2d). In seven plants of the resistant clonal line, isolated lesion patches occurred beyond the lesion that extended from the inoculation point (Figure 6.2e).

No lesions developed in the wounded plants not inoculated with *P. cinnamomi*.

6.3.1.2 Acropetal lesion extension

The effect of temperature on lesion extension in stems of *E. marginata* at 9-10 days after inoculation with *P. cinnamomi* was significant in Experiment 2, and was close to significance in Experiment 1 (Table 6.2). At harvest time, lesions were continuing to extend up to 5 mm per day in the 28°C treatment and were up to six times longer

than at 20°C (Figure 6.3). Lesion extension at 20°C was limited, with mean daily extensions of approximately 0.1 mm (Figure 6.3). Genotype did not affect lesion extension significantly (Table 6.2).

Phosphite treatment had a significant effect on lesion extension at 7 days after spraying, with a reduction in lesion extension evident by 2 days after treatment in plants at 28°C (Figure 6.3b and Table 6.2). However, lesion extension was not halted in plants treated with 5 g phosphite/L, particularly in the susceptible clonal line at 28°C (Figure 6.3b). Any effect of phosphite on lesion extension at 20°C was not apparent, except that lesions were 1.5-1.8 mm shorter in the phosphite-treated plants.

6.3.1.3 Colonisation by *P. cinnamomi* beyond the lesion

P. cinnamomi was isolated from all inoculated plants and lesions, including the lesion patches described in Section 6.3.1. Colonisation of the stem by *P. cinnamomi* beyond the lesion was generally no further than 1 cm, except in plants of the resistant clonal line (Figure 6.4), in which there was a significantly greater extension beyond the lesion than in the susceptible line (Table 6.2). Temperature affected the extension beyond the lesion significantly (Table 6.2), with generally greater extensions at 20°C than at 28°C (Figure 6.4). Phosphite-treated plants had shorter extensions beyond the lesion than untreated plants, particularly at 20°C (Figure 6.4b).

6.3.1.4 Circumferential lesion spread

Temperature and genotype each had a significant effect on the extent of circumferential spread of lesions after inoculation with *P. cinnamomi* in Experiment 2, but not in Experiment 1 (Table 6.2). In both experiments, the lesion spread at 20°C was limited and static by 4 days after inoculation (Figure 6.5). At 28°C, lesions

continued to spread tangentially for the duration of Experiment 2, except in plants treated with phosphite (Figure 6.5). Although the plants treated with phosphite had reduced circumferential lesion spread in comparison with plants not receiving phosphite treatment, the difference was not significant (Table 6.2). At 20 and 28°C, the extent of circumferential spread of lesions was the least in the resistant clonal line (Figure 6.5).

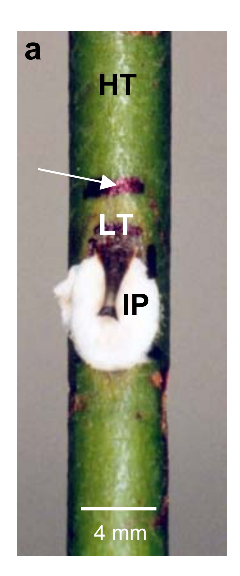
One plant was girdled at 20°C, while up to 94% of plants were girdled at 28°C (Table 6.3). In Experiment 2, significantly more plants were girdled in the SS/0 treatment than in any of the other treatments at 28°C. The RR/5 treatment had significantly fewer plants girdled at 28°C than plants in the other treatments. Within each genotype, phosphite significantly reduced the percentage of girdled plants (Table 6.3).

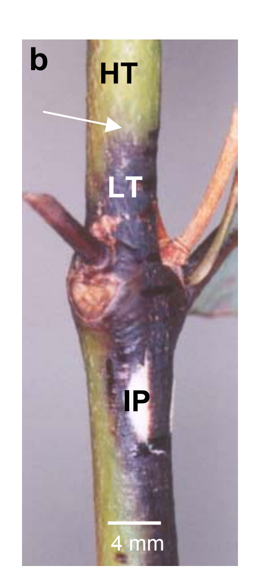
6.3.1.5 Wilting/death

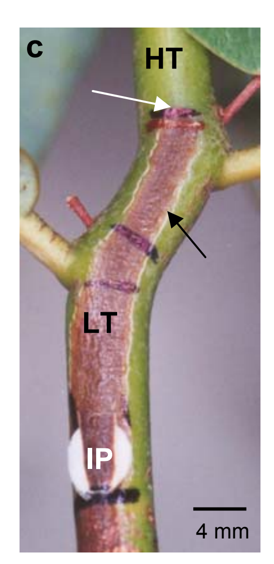
No wilting or death of plants was observed at 20°C. At 28°C, up to 50% of plants were noted to wilt (Table 6.3), with the time taken to wilt ranging from 1-6 days after girdling. Some plants had not wilted by 6 days after girdling. There was a significant difference between the number of girdled plants and the number of wilted plants in the SS/0 treatment (Table 6.3), with only 20-47% of girdled plants subsequently wilting. Genotype and phosphite had little effect on the number of wilted plants, except for a significant difference between the SS/0 and RR/5 treatments (Table 6.3).

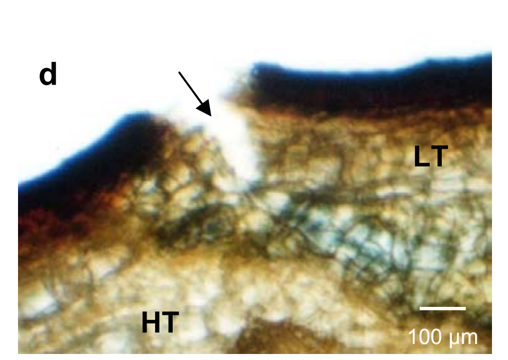
6.3.1.6 Stem growth

Temperature had a significant (P=0.02) effect on the change in stem diameter in uninoculated plants at 7 days after wounding in Experiment 1, with increases of 0.12±0.03 and 0.25±0.05 mm at 20 and 28°C, respectively.


a
HT
LT
IP
4 mm


b
HT
LT
IP
4 mm


c
HT
LT
IP
4 mm


d
LT
HT
100 µm


e
HT
*
HT
LT
IP
4 mm

Figure 6.2: Lesions in stems of resistant (RR) and susceptible (SS) plants of *Eucalyptus marginata* at 9 days after inoculation with *Phytophthora cinnamomi*. Plants were sprayed with 0 and 5 g phosphite/L at 2 days after inoculation and incubated at 20 and 28°C. The pen markings indicate the progression of lesions up the stem from the inoculation point (IP), while the white arrowheads indicate the lesion fronts.

a: Limited lesion extension in RR/5 treatment at 20°C. The lesioned tissue (LT) is pale yellow.

b: Dark brown lesion formed in SS/5 treatment at 28°C. The lesion margin is not well-defined.

c: Lesioned tissue in RR/5 treatment at 28°C, with a well-defined white/yellow lesion margin.

d: Transverse section of white/yellow lesion margin shown in **c**. The lesioned tissue is breaking away from the healthy tissue (HT) in the epidermis and cortex (indicated by a black arrowhead).

e: Isolated lesion patch in treatment RR/0 at 28°C (indicated by an asterisk). The majority of tissue between the lesion patch and the previous lesion front (indicated by a circle) appears healthy.

Table 6.2: Summary of MANOVA[A] for the effect of temperature (20 and 28°C), plant genotype [resistant (RR) and susceptible (SS)] and phosphite treatment (0 and 5 g phosphite/L) on lesion lengths, extension beyond the lesion, total colonisation and circumferential lesion spread. Assessments were made at 10 (Experiment 1) or 9 (Experiment 2) days after inoculation of *Eucalyptus marginata* stems with *Phytophthora cinnamomi*.

Independent variable	Lesion extension	Extension beyond the lesion	Total colonisation	Circumferential spread
Experiment 1				
Temperature	0.06[B]	0.01*[B]	0.02*[B]	0.07[B]
Plant genotype	0.6	0.001*	0.2	0.1
Experiment 2				
Temperature	<0.001*	0.04*	<0.001*	<0.001*
Plant genotype	0.6	0.003*	0.5	0.02*
Phosphite treatment	0.008*	0.002*	0.002*	0.09
Temperature x plant genotype	0.8	0.3	0.6	0.1
Temperature x phosphite treatment	0.01*	0.007*	0.08	0.08
Plant genotype x phosphite treatment	0.6	0.2	0.4	0.5
Temperature x plant genotype x phosphite treatment	0.6	0.2	0.8	0.6

[A] Statistical analysis of data presented in Figures 6.3-6.5.

[B] P values from MANOVA. * indicates that effects are significant when α = 0.05.

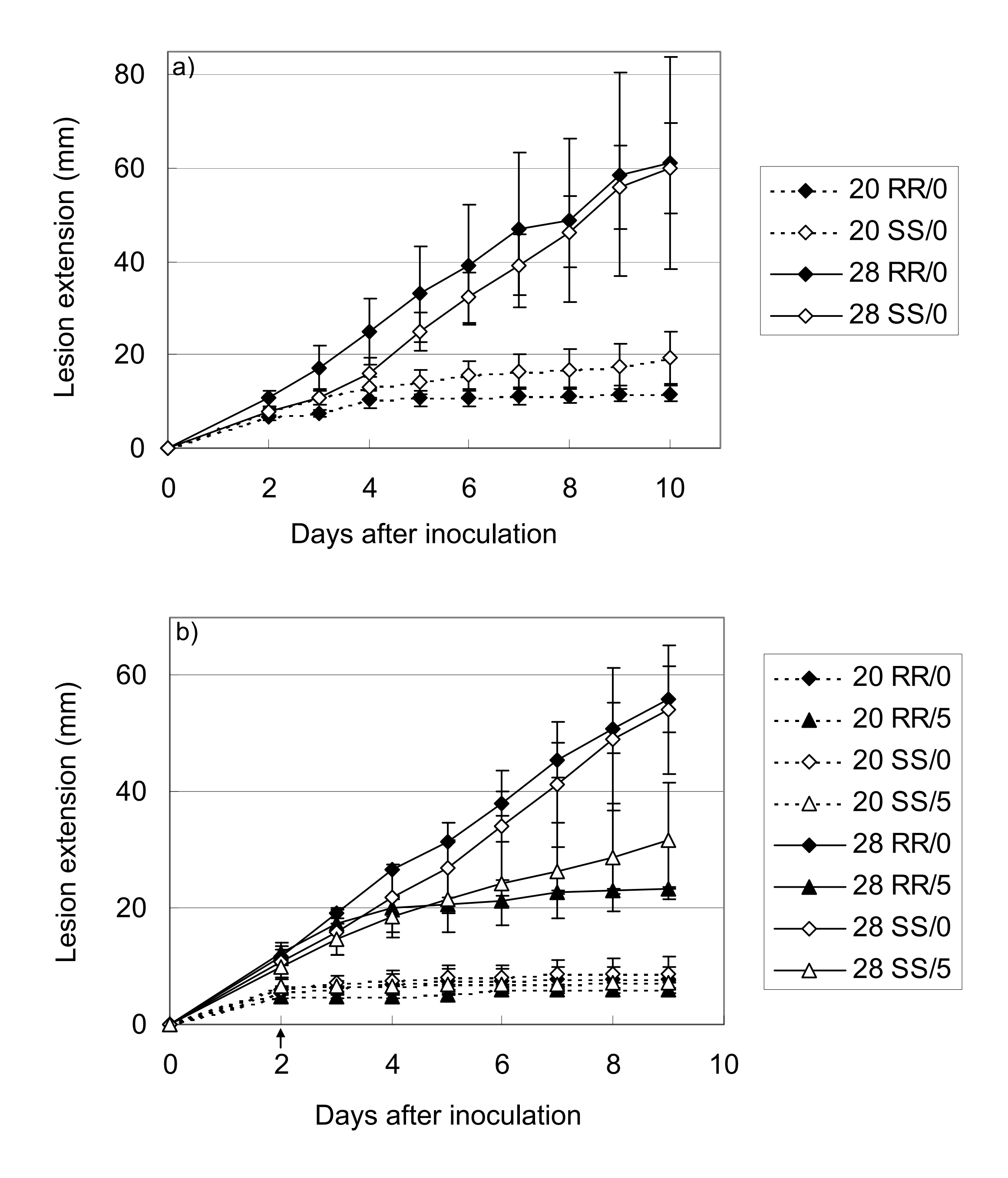
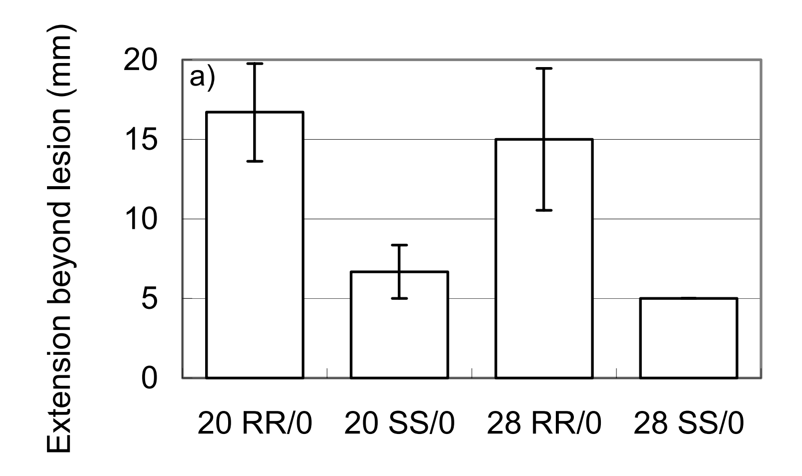
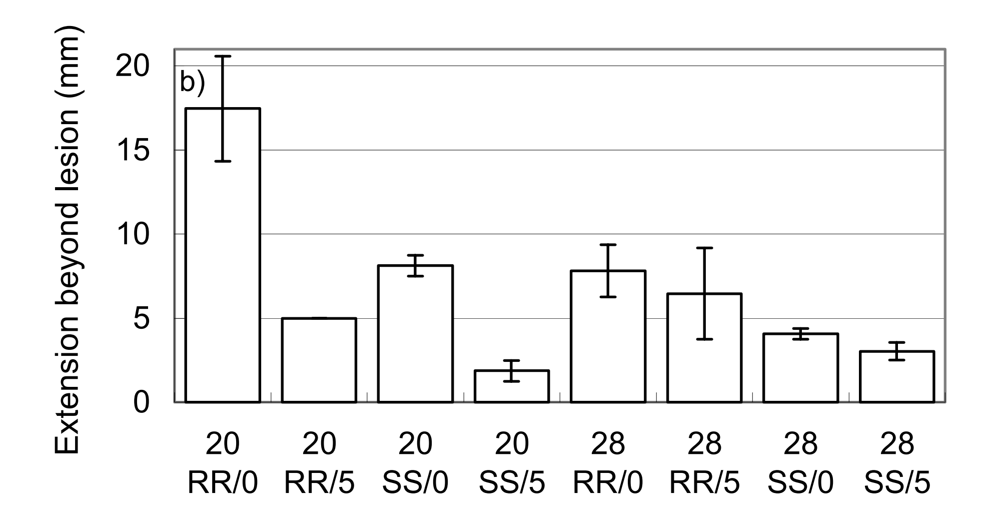


Figure 6.3: Daily lesion extension in stems of *Eucalyptus marginata* [resistant (RR) and susceptible (SS) genotypes] in a) Experiment 1 and b) Experiment 2 after inoculation with *Phytophthora cinnamomi*. Plants were incubated at 20 or 28°C. In Experiment 2, plants were sprayed with 0 or 5 g phosphite/L at 2 days after inoculation (indicated by an arrow) (refer to legends for symbols). Values are means of repeated measurements of six (a) to sixteen (b) plants ± the standard errors of the means.

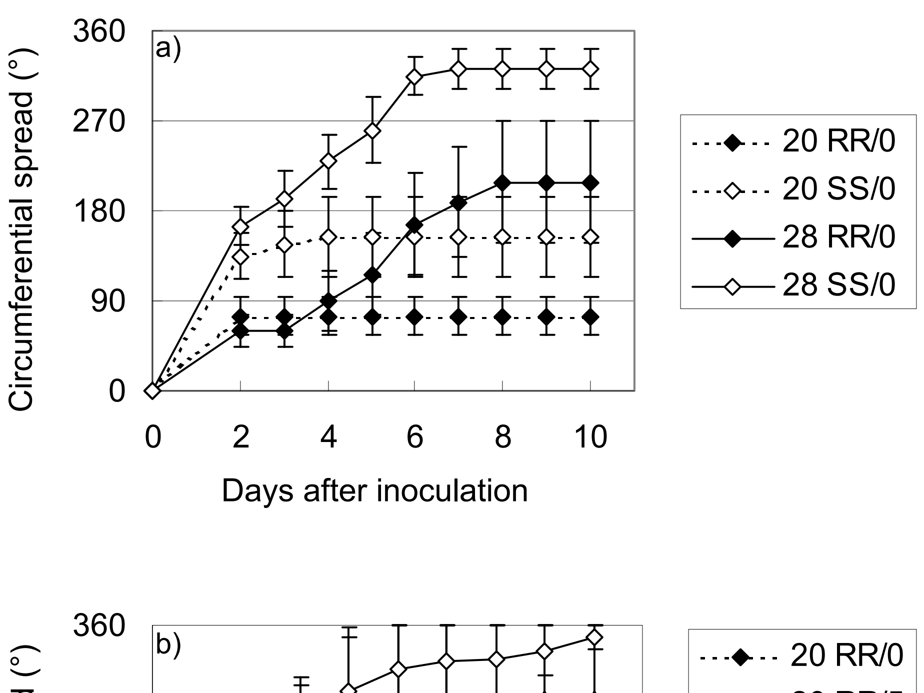


Temperature (°C), clonal line/phosphite treatment (g/L)



Temperature (°C), clonal line/phosphite treatment (g/L)

Figure 6.4: Extent of colonisation by *Phytophthora cinnamomi* beyond the lesion in stems of *Eucalyptus marginata* [resistant (RR) and susceptible (SS) genotypes] at a) 10 days (Experiment 1) and b) 9 days (Experiment 2) after inoculation. In Experiment 2, plants were sprayed with 0 or 5 g phosphite/L at 2 days after inoculation. Values are means of six (a) and sixteen (b) plants ± the standard errors of the means.



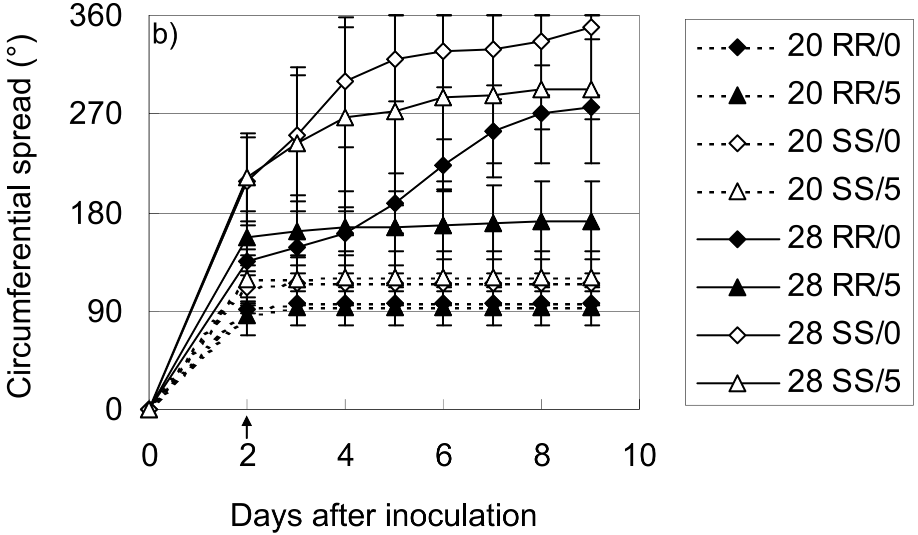


Figure 6.5: Daily circumferential spread of lesions in stems of *Eucalyptus marginata* [resistant (RR) and susceptible (SS) genotypes] in a) Experiment 1 and b) Experiment 2 after inoculation with *Phytophthora cinnamomi*. Plants were incubated at 20 or 28°C. In Experiment 2, plants were sprayed with 0 or 5 g phosphite/L at 2 days after inoculation (indicated by an arrow) (refer to legends for symbols). Values are means of repeated measurements of six (a) to sixteen (b) plants ± the standard errors of the means.

Table 6.3: Incidence of girdling and wilting/death in resistant (RR) and susceptible (SS) clonal lines of *Eucalyptus marginata* up to 10 days after inoculation with *Phytophthora cinnamomi*. Plants were incubated at either 20 or 28°C and treated with either 0 or 5 g phosphite/L at 2 days after inoculation.

Genotype/ phosphite treatment	Girdled (% of plants)[A]		Wilted/dead (% of plants)	
	Temperature (°C)			
	20	28	20	28
Experiment 1				
RR/0	0a[B]	50a[B]	0a[B]	50a[B]
SS/0	17a	83a*	0a	17a*
Experiment 2				
RR/0	0a	50b	0a	31ab
RR/5	0a	6c	0a	6b
SS/0	0a	94a*	0a	44a*
SS/5	0a	56b	0a	19ab

[A] Girdling = 360° circumferential spread of lesion.

[B] Within column for each experiment, values followed by the same letter are not significantly different, based on 95% confidence intervals.

* indicates a significant difference between incidences of girdling and wilting within a temperature and genotype/phosphite treatment, based on 95% confidence intervals.

6.3.1.7 Histology of wound response in uninoculated stems

The wounds inflicted with the razor blade were shallow, extending slighter deeper than the primary phloem. The observed responses to the wounding occurred in tissues up to 300 μm away from the wound edge. Phosphite treatment did not significantly affect the responses to wounding.

Pre-existing cells

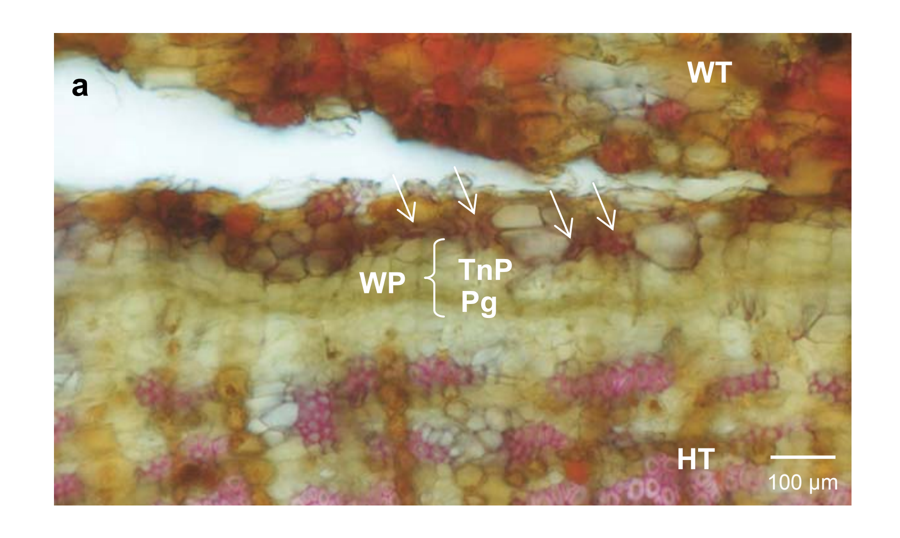
At 7 days after wounding, the walls of cells that existed prior to the time of injury reacted positively to PHCl (Figure 6.6a) but not to SBB, indicating the presence of lignin and absence of suberin. In samples where lignin was only just detectable, it was often deposited in the corners of cell walls and immediately internal to phloem fibres. At 20°C, lignin was either not detected or was present at very low levels in 75-83% of samples. Similarly, low levels of lignin were observed in 92% of samples from the RR clonal line at 28°C. More extensive lignin deposition was observed in the remaining 17-25% of samples at 20°C and in 92% of plants in the SS clonal line at 28°C.

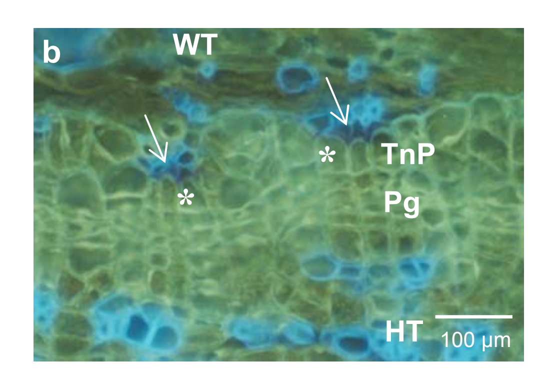
Periderm formation

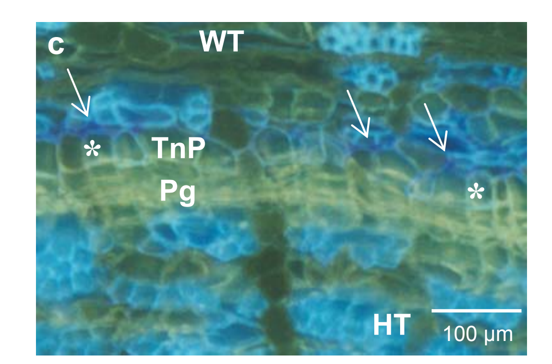
A meristematic layer formed internal to the PHCl-positive tissue (Figures 6.6a-c) in all samples at 7 days after wounding, with the exception of 2/12 plants in the RR genotype at 28°C (Figure 6.7a). In 50-100% of plants at 20°C and 67-100% plants at 28°C, autofluorescent cells were visible external to and in radial alignment with the meristematic layer (Figures 6.6c+d and 6.7c). The fluorescent and staining properties of these cells indicated that they were suberised. The outer wall of the outermost layer of suberised cells was often dome-shaped and thickened with lignin. These cells are considered to be the first phellem produced by the phellogen of the wound periderm (WP). While only one layer of cells had differentiated into phellem

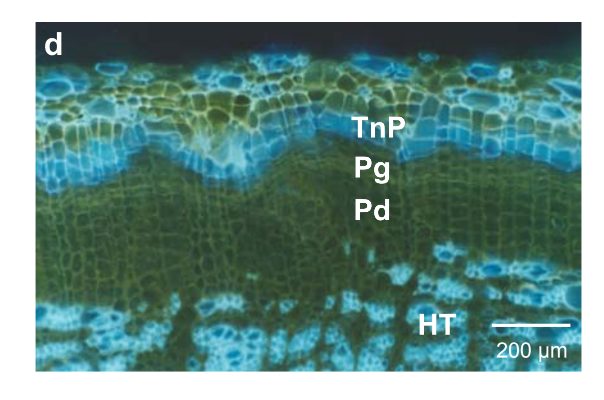
in the plants incubated at 20°C (Figures 6.6c), up to three phellem layers were visible in 83 and 50% of samples incubated at 28°C in the SS and RR clonal lines, respectively. Internal to the phellogen, up to seven layers of phelloderm cells were visible in the SS genotype at 28°C, distinguishable from older parenchyma cells by their radial alignment with the phellogen and phellem cells (Figure 6.6d+e). Phelloderm cells were limited to two or three cell layers in the RR genotype at 28°C and were not distinguishable in plants incubated at 20°C.

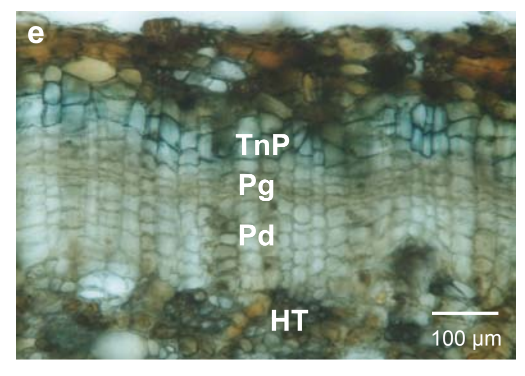
In one SS plant incubated at 28°C, a distinctive single layer of cubical phellem cells had formed internal to the first-formed phellem (Figure 6.6f). Similar phellem cells were observed in 3/12 plants of the RR genotype incubated at 28°C (Figure 6.6g). The cubical phellem cells were in radial alignment with the first-formed phellem cells and the phelloderm cells.

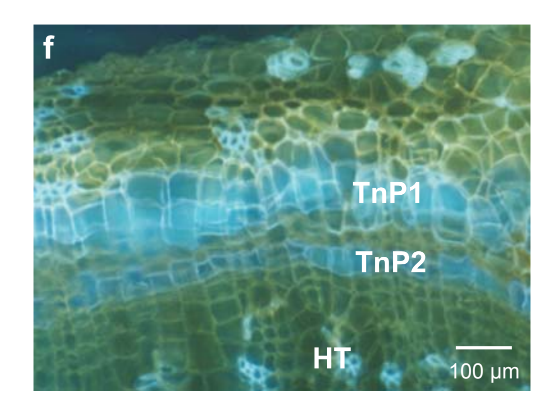

a
WT
WP
TnP
Pg
HT
100 µm


b
WT
TnP
Pg
HT
100 µm


c
WT
TnP
Pg
HT
100 µm


d
TnP
Pg
Pd
HT
200 µm


e
TnP
Pg
Pd
HT
100 µm


f
TnP1
TnP2
HT
100 µm

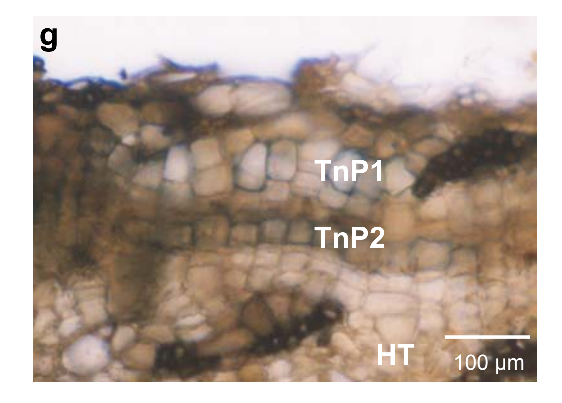

g
TnP1
TnP2
HT
100 µm

Figure 6.6: Response to shallow wounding with a razor blade in stems of resistant (RR) and susceptible (SS) plants of *Eucalyptus marginata* at 7 days after wounding. Plants were sprayed with 0 and 5 g phosphite/L immediately after wounding and incubated at 20 and 28°C.

a: Deposition of lignin (arrowheads) in cells adjacent to wounded tissue (WT) in the RR/5 treatment at 28°C. A wound periderm (WP) is forming internal to the lignified tissue, consisting of a phellogen (Pg) and thin-walled phellem (TnP). The transverse section has been stained with Phloroglucinol + HCl (PHCl) and viewed with brightfield light.
b+c: Early stages of WP formation in treatments SS/0 (**b**) and RR/0 (**c**) at 20°C. Several of the first-formed TnP cells are dome-shaped (asterisk) and are only weakly autofluorescent when observed in blue light. Some quenching of lignin autofluorescence is visible (arrowheads) in these sections stained with PHCl.
d+e: Strong autofluorescence of TnP (**d**) in the SS/0 treatment at 28°C, with cell walls reacting positively for suberin in a section stained with Sudan Black B (**e**). Several layers of TnP and phelloderm (Pd) have been produced from the Pg.
f+g: A single layer of cubical TnP cells (TnP2) formed two to three cell layers internal to the first-formed TnP layers (TnP1) in the SS/0 and RR/5 treatments at 28°C.

Further abbreviations (for anything not previously mentioned in text): HT = healthy tissue.

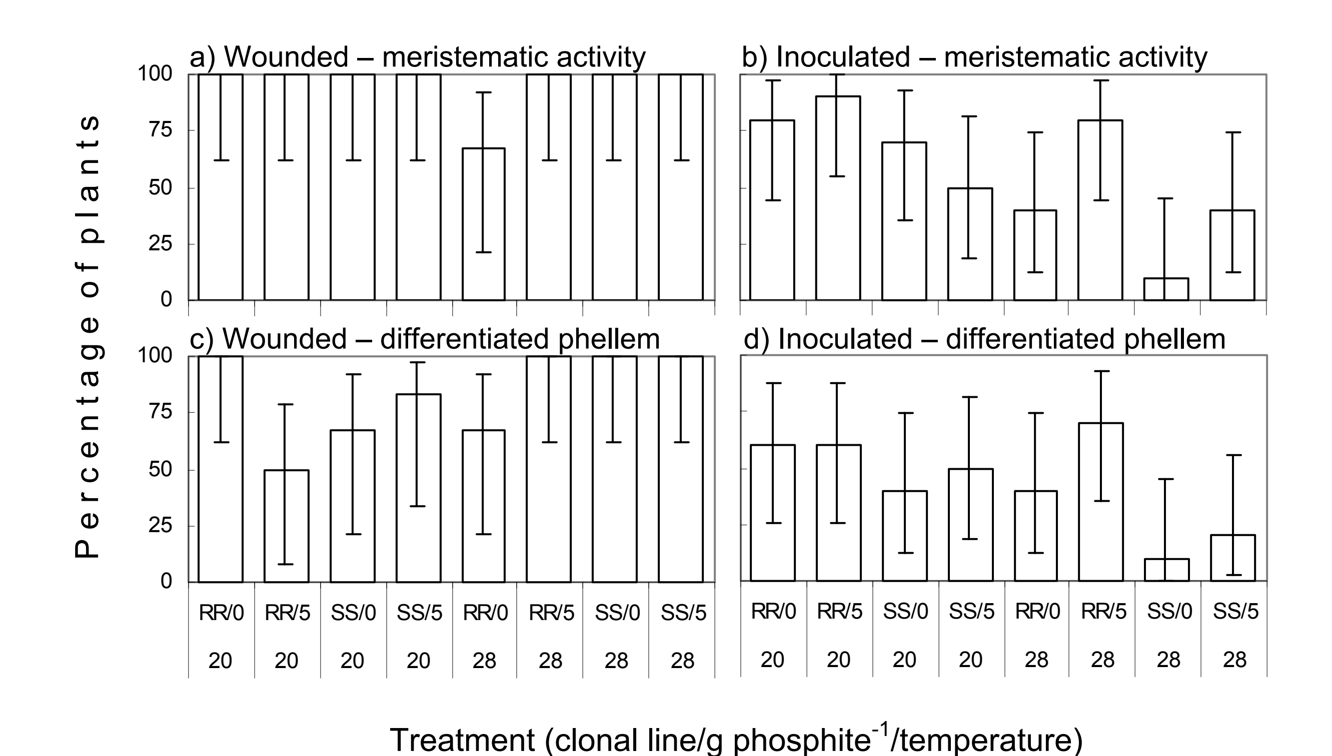


Figure 6.7: Percentage of resistant (RR) and susceptible (SS) plants of *Eucalyptus marginata* with observed meristematic activity (a+b) and phellem differentiation (c+d) adjacent to a shallow wound (a+c) and at the lesion front in plants inoculated with *Phytophthora cinnamomi* (b+d). Plants were incubated at 20 and 28°C and sprayed with 0 or 5 g phosphite/L immediately after wounding (a+c) or 2 days after inoculation with *P. cinnamomi* (b+d). Assessments were made 7 days after wounding (a+c) and 9 days after inoculation (b+d). Bars represent the 95% confidence interval for each value (n=6-10).

6.3.1.8 Histology of response in stems inoculated with *P. cinnamomi*

Tissue damage

In sections cut at the lesion edge, tissues external to the wood were necrotic in all plants at 9 days after inoculation with *P. cinnamomi*. Damage to the xylem was most extensive at 28°C, with discoloured wood and occluded vessels (Figure 6.8a) observed in all plants of the SS clonal line and 55% of the RR plants. At 20°C, xylem damage was evident in 25 and 45% of plants in the RR and SS clonal lines, respectively.

The xylem was also discoloured and occluded in sections cut from beyond the lesion edge in 25% (20°C) and 88% (28°C) of samples examined. In addition, inner bark necrosis/damage was visible beyond the lesion edge in 13% (20°C) and 63% (28°C) of samples. Although plants of the RR genotype incubated at 20°C were suspected to have lesion patches near the vascular cambium (VC) when examined macroscopically, the dark patches were instead pockets of kino.

Wound responses

The response to wounding in uninoculated plants was similar to that described for Experiment 1. Conversely, plants inoculated with *P. cinnamomi* did not exhibit any response to the wound inflicted with the razor blade. Responses were observed at the lesion edge.

Pre-existing cells

The walls of extant cells reacted positively to both PHCl and SBB, indicating the presence of both lignin and suberin. At 20°C in all treatments and 28°C in the RR/5 treatment, walls of pre-existing cells were thickened with lignin and suberin in 90-100% of plants. In the remaining treatments at 28°C, 20-60% of plants were noticeably lignified and 20-40% were suberised. Figures 6.8b-e illustrate the

presence of PHCl-positive tissue, while Figure 6.8f shows the staining responses of cell walls to SBB.

Periderm formation

Temperature significantly affected the development of a meristematic layer adjacent to extant cells in inoculated plants not treated with phosphite, but had no effect on the incidence of division in phosphite-treated plants (Figure 6.7b). Within the 20°C treatment, the incidence of division was not significantly affected by phosphite treatment or genotype. At 28°C, meristematic activity was reduced in the susceptible line and RR/0 treatment in comparison with the RR/5 treatment (Figure 6.7b). Figure 6.9a shows an early stage of periderm formation, with some meristematic activity but no differentiation of phellem cells.

Phellem production was observed in 40-60% of plants at 20°C and 10-70% of plants at 28°C, with the maximum incidence in the RR/5 treatment (Figure 6.7d). As described in Section 6.3.7, the phellem cells were suberised, with a dome-shaped and lignified outer wall (Figure 6.9b+c). There were no samples in which there were clearly two layers of phellem or any phelloderm distinguishable from the phellogen.

Extent of suberisation

The proportion of lesion edge with adjacent suberised cells was reduced in the RR/0, SS/0 and SS/5 treatments at 28°C in comparison with 20°C (Table 6.4). The extent of suberisation observed in the RR/5 treatment was not affected by temperature. There was considerable variation between treatments at 28°C, with weak and strong responses in the SS/0 and RR/5 treatments, respectively. This contrasted with the homogeny of responses at 20°C.

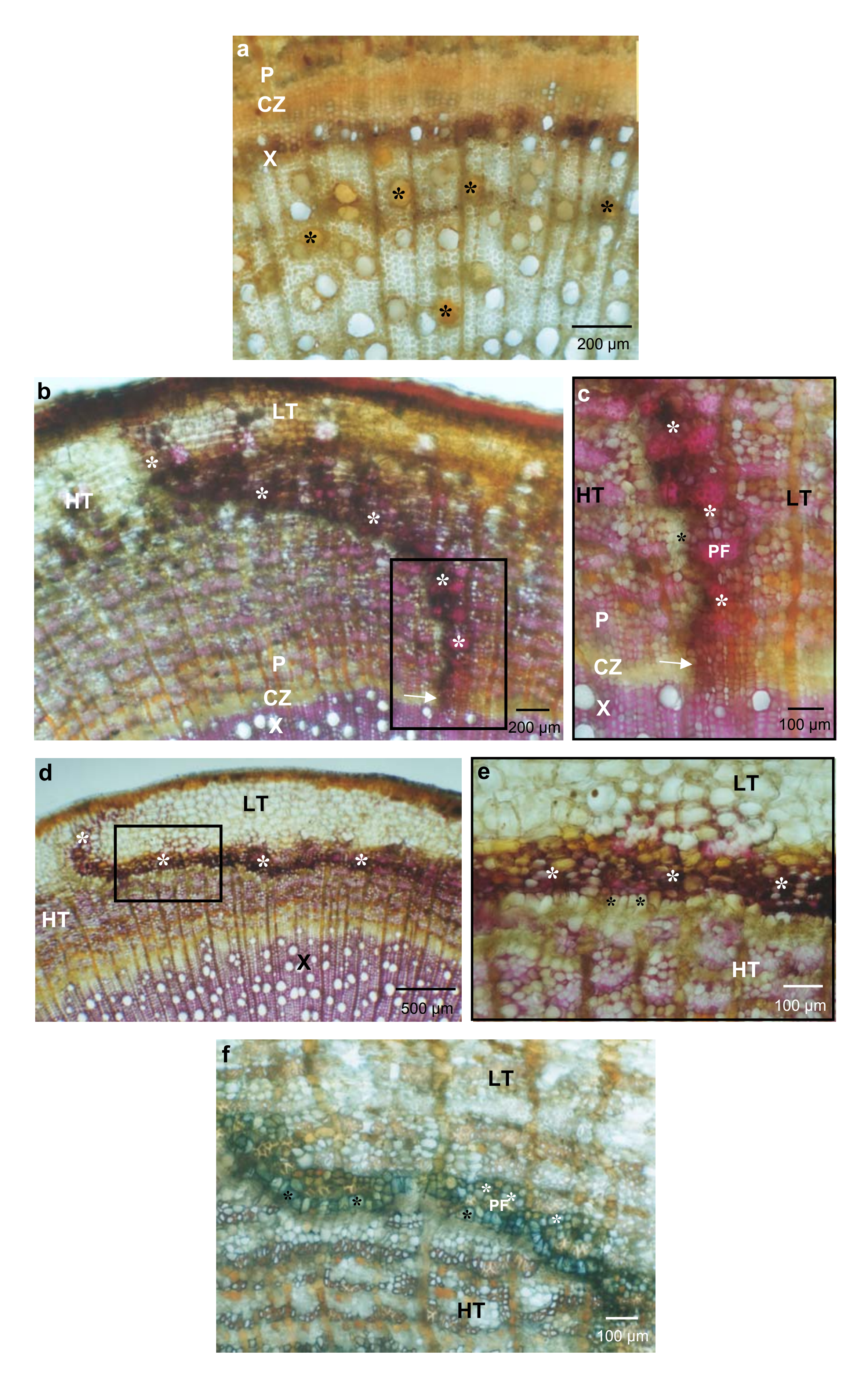

a
P
CZ
X
200 µm
b
LT
HT
P
CZ
X
200 µm
c
HT
LT
PF
P
CZ
X
100 µm
d
LT
HT
X
500 µm
e
LT
HT
100 µm
f
LT
PF
HT
100 µm

Figure 6.8: Responses in stems of resistant (RR) and susceptible (SS) plants of *Eucalyptus marginata* at the lesion front 9 days after inoculation with *Phytophthora cinnamomi*. Plants were sprayed with 5 g phosphite/L at 2 days after inoculation and incubated at 20 and 28°C. The transverse sections are all viewed with brightfield light.

a: Discoloured wood and occluded xylem vessels (asterisks) in the SS/5 treatment at 28°C. The section is unstained.

b-e: Lignin deposition (white asterisks) at the junction of healthy tissue (HT) and lesioned tissue (LT) in RR/5 treatment at 28°C (**b+c**) and SS/5 treatment at 20°C (**d+e**). Staining is continuous in **b** from the epidermis and through the cambial zone (CZ). A higher magnification of the area outlined in **b** shows the response in the CZ (indicated by arrowheads) and external phloem (P) tissue more clearly (**c**). Likewise, **e** shows the outlined area of **d** at a higher magnification. Dome-shaped thin-walled phellem cells (TnP – black asterisks) of a wound periderm are visible in healthy tissue adjacent to the lignin deposition (**c+e**). Sections are stained with Phloroglucinol + HCl.

f: Suberin deposition in walls of extant cells (white asterisks) and TnP from a wound periderm (black asterisks) in the RR/5 treatment at 20°C. The section is stained with Sudan Black B.

Further abbreviations (for anything not previously mentioned in text): X = xylem and PF = phloem fibres.

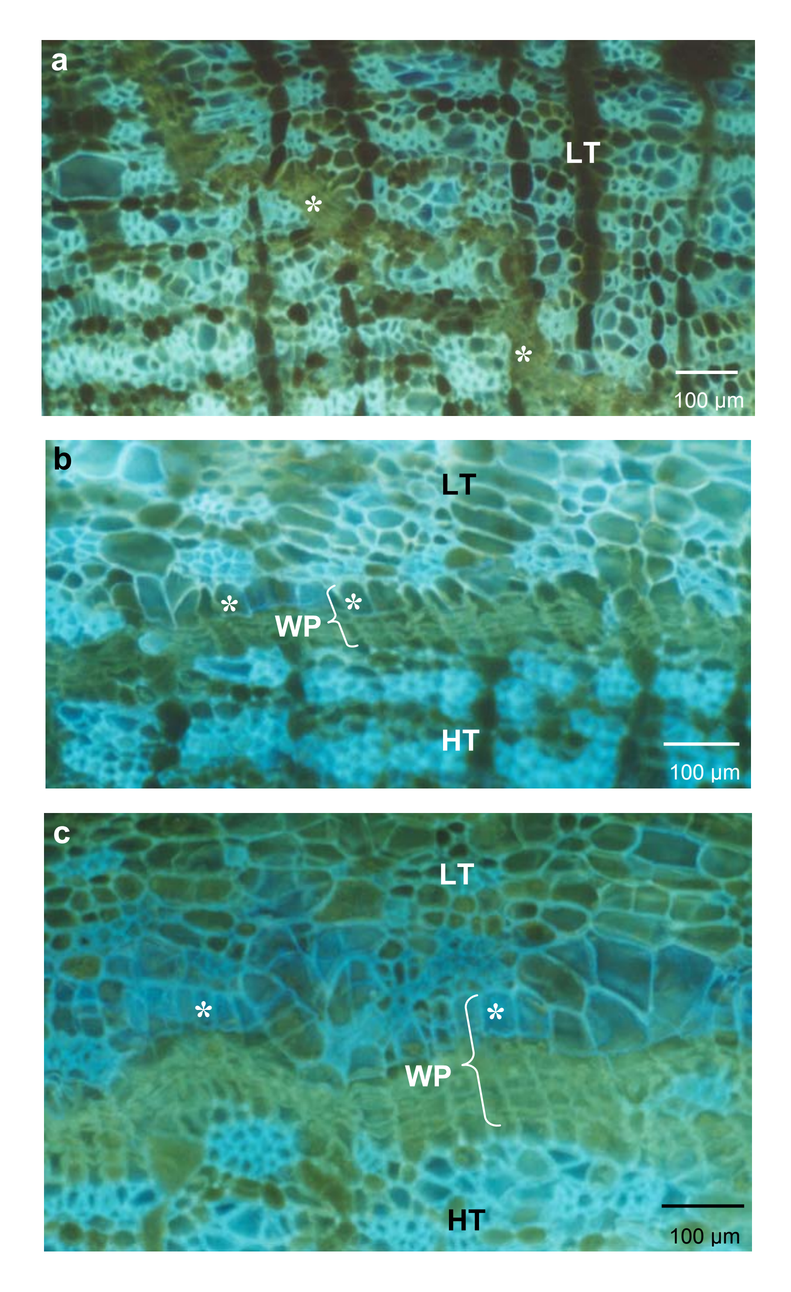


Figure 6.9: Wound periderm (WP) formation in stems of resistant (RR) and susceptible (SS) plants of *Eucalyptus marginata* at the lesion front 9 days after inoculation with *Phytophthora cinnamomi*. Plants were sprayed with 0 and 5 g phosphite/L at 2 days after inoculation and incubated at 20°C. All transverse sections are unstained and viewed under blue light.

a: Some evidence of meristematic activity (asterisks) in the SS/0 treatment.

b: An early stage in the development of a WP in the RR/5 treatment, with weak autofluorescence in a few dome-shaped thin-walled phellem cells (TnP – asterisks).

c: WP with strongly autofluorescent TnP cells (asterisks) in the SS/5 treatment.

Further abbreviations (for those not previously mentioned in text): LT = lesioned tissue and HT = healthy tissue.

Table 6.4: Mean extent of suberisation along bark lesion edge in stems of resistant (RR) and susceptible (SS) clonal lines of *Eucalyptus marginata*. Plants were incubated at 20 or 28°C (Experiment 2) and 23 or 24°C (Experiment 3), treated with either 0 or 5 g phosphite/L at 2 days after inoculation with *Phytophthora cinnamomi* and harvested at 9 days after inoculation (n=10 in Experiment 2 and n=5 in Experiment 3). NT = not tested.

Ratings for extent of suberisation in transverse sections treated with SBB:

0 = suberin not detected, 1 = 1-50%, and 2 = 51-100% lesion edge with adjacent suberised cells.

Genotype/ phosphite treatment	Mean extent of suberisation rating			
	Temperature (°C)			
	20	23	24	28
RR/0	1.7	1.2	1.2	0.6
SS/0	1.6	0.6	0.6	0.2
RR/5	1.7	NT	NT	1.8
SS/5	1.4	NT	NT	0.5

6.3.1.9 Responses of uninoculated versus inoculated tissues

Figure 6.10 summarises the incidences of responses observed in wounded and inoculated tissues at 20 and 28°C. The histochemical responses of extant cells occurred in more of the inoculated plants than the uninoculated plants, with the exception of the SS clonal line at 28°C. A periderm was observed in fewer inoculated than uninoculated plants in the SS clonal line. After wounding, the greatest incidence of lignin deposition and phellem differentiation occurred in the SS/0 and SS/5 treatments at 28°C. These treatments resulted in the lowest incidence of the observed histological responses in plants inoculated with *P. cinnamomi*.

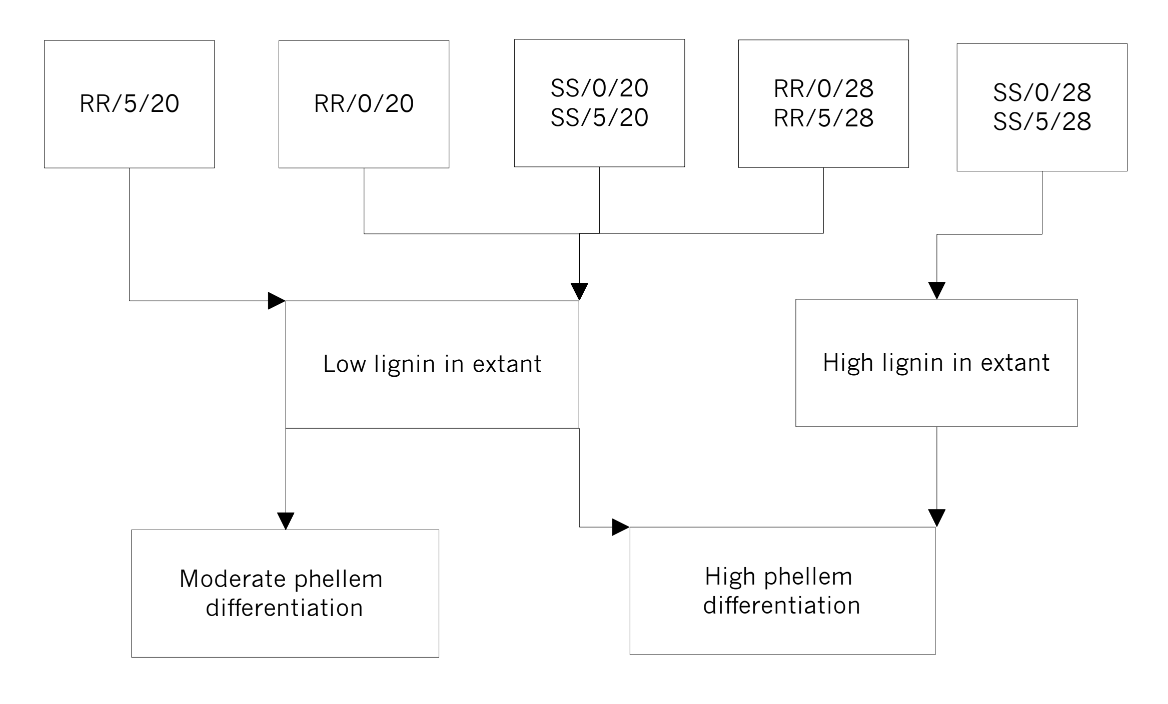
6.3.2 Experiment 3: 23 and 24°C

Lesion lengths and circumferential spread were generally intermediate to those observed at 20 and 28°C (Figures 6.11a+c – c.f. Figure 6.3 and 6.5). Neither acropetal extension nor circumferential spread of *P. cinnamomi* lesions was significantly affected by temperature (P=0.6, 0.6) or plant genotype (P=0.4, 0.2). There was, however, a greater difference in lesion extension and spread between genotypes at 23°C than at 24°C (Figures 6.11a+c), with the smallest lesions observed in the resistant clonal line.

The extension of *P. cinnamomi* beyond the lesion was significantly affected by temperature and plant genotype (P=0.03), with the largest extension in the resistant clonal line at 24°C (Figure 6.11b).

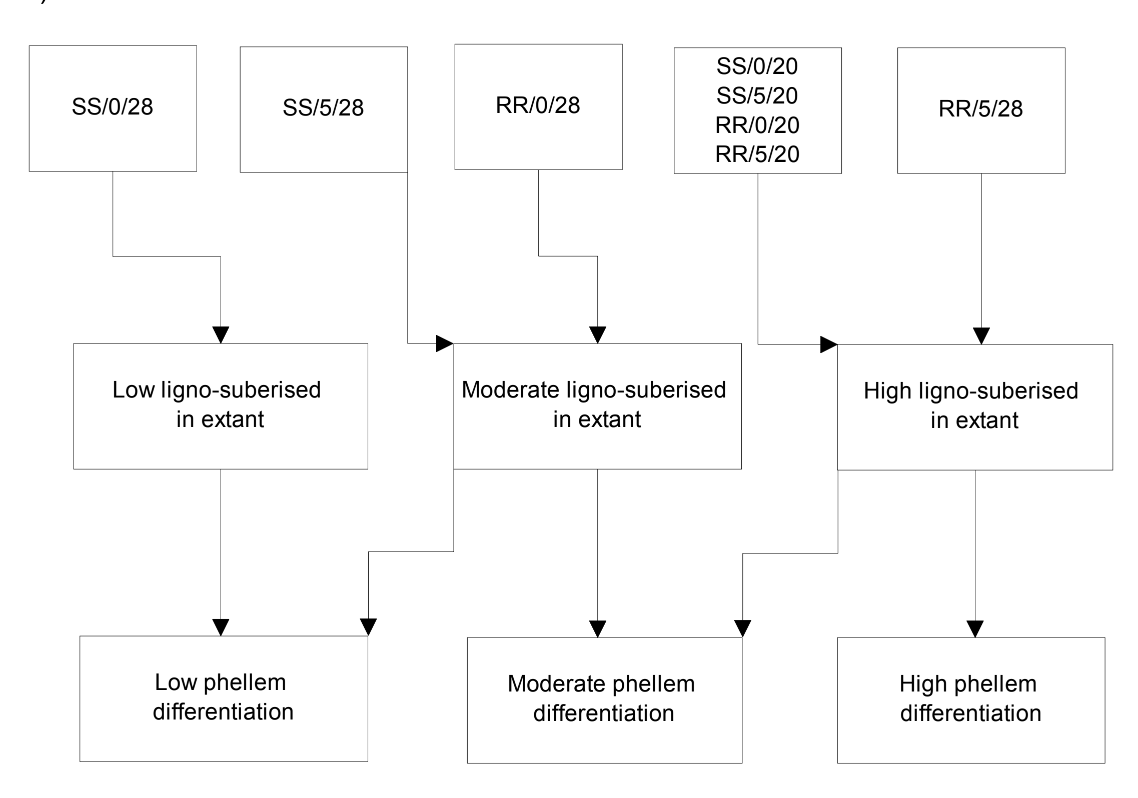
The extents of suberisation at the lesion front were identical at 23 and 24°C and intermediate to the responses observed at 20 and 28°C (Table 6.4). The resistant clonal line exhibited the strongest responses at all temperatures tested.

a)



RR/5/20
RR/0/20
SS/0/20
SS/5/20
RR/0/28
RR/5/28
SS/0/28
SS/5/28
Low lignin in extant
High lignin in extant
Moderate phellem differentiation
High phellem differentiation

b)

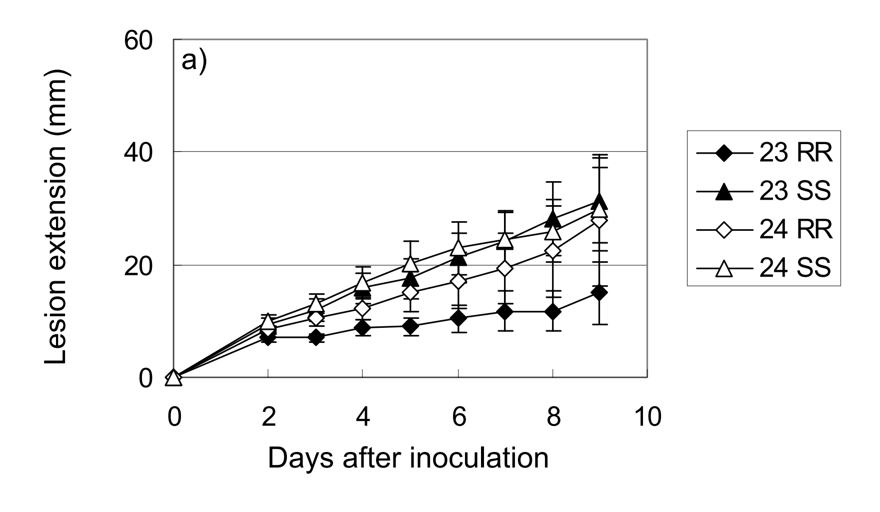


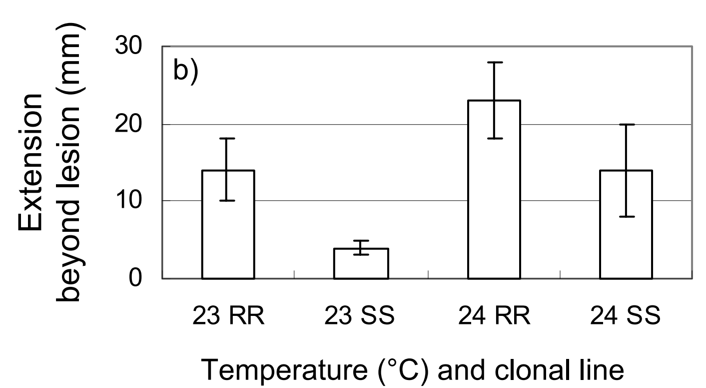
SS/0/28
SS/5/28
RR/0/28
SS/0/20
SS/5/20
RR/0/20
RR/5/20
RR/5/28
Low ligno-suberised in extant
Moderate ligno-suberised in extant
High ligno-suberised in extant
Low phellem differentiation
Moderate phellem differentiation
High phellem differentiation

Figure 6.10: Summary of the incidence of histological responses in resistant (RR) and susceptible (SS) plants of *Eucalyptus marginata* sprayed with 0 and 5 g phosphite/L and incubated at 20 and 28°C. Plants were assessed for histochemical staining in extant cells and wound periderm formation (with differentiated phellem) adjacent to a) an uninoculated wound (7 days after wounding and spraying) and b) lesioned tissue at the lesion front (9 days after inoculation with *Phytophthora cinnamomi*, sprayed 2 days after inoculation).

Incidences are grouped in these categories: low = 0 – 30% of plants positive, moderate = 30 – 70% of plants positive and high = 70 – 100% of plants positive (n=6-10).

Each treatment follows through the same side of the box at which the arrow enters. For example, the RR/5/20 treatment follows through from low lignin in extant cells to moderate phellem differentiation.





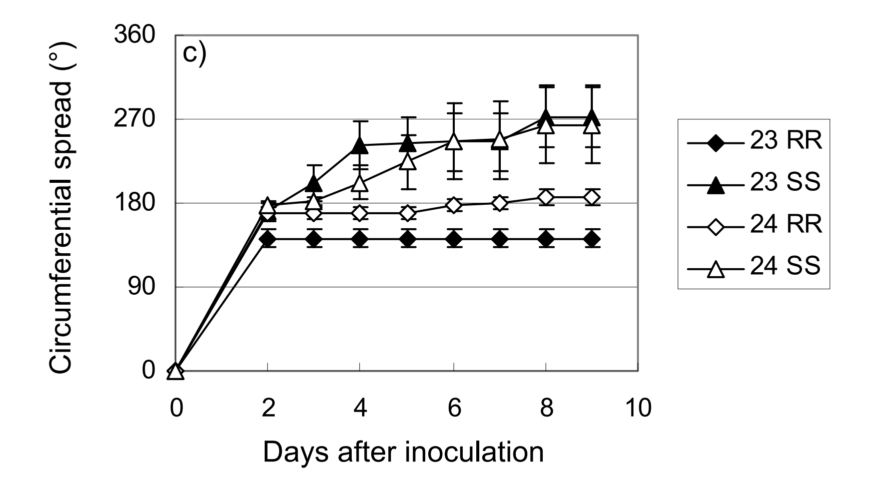


Figure 6.11: a) Lesion extension, b) extension beyond lesion and c) circumferential spread of *Phytophthora cinnamomi* in stems of RR (resistant) and SS (susceptible) clonal lines of *Eucalyptus marginata* (Experiment 3). Plants were incubated at 23 or 24°C for 9 days after inoculation and were not treated with phosphite. Values are means of five plants ± the standard errors of the means.

6.4 Discussion

Temperature affected the *E. marginata*-*P. cinnamomi* interaction in the same way as described by Zentmyer (1981), Grant and Byrt (1984), Shearer *et al*. (1987) and Hüberli *et al*. (2002), with greater disease development at 28°C than at 20°C. Although the clonal lines 1J30 and 11J402 were previously classified as resistant (RR) and susceptible (SS) according to stem lesion lengths and this was confirmed with field mortality results (McComb *et al*., 1990), there was no difference between genotypes in plant mortality or in the length of lesions that developed after inoculation with *P. cinnamomi* in the current study. At 28°C, which is optimum for the *in vitro* growth of the *P. cinnamomi* isolate used (Hüberli, 1995), the conditions favoured the pathogen and did not allow general resistance to be expressed. As observed in Chapter 5, plants of both the resistant and susceptible genotypes were able to compartmentalise wounds in the absence of the pathogen. Periderm formation was more advanced at 28°C than at 20°C, which agrees with the observed effects of temperature in other studies of wound responses (Garrod *et al*., 1982; Biggs and Northover, 1985; Biggs, 1986; Doster and Bostock, 1988). Despite this potential for a response at 28°C, the genotypes were equally ineffective at responding to colonisation by *P. cinnamomi*. The presence of the pathogen disrupted the hosts' responses, as has been reported for several other host-pathogen interactions (Biggs, 1984b; Hebard *et al*., 1984; Wisniewski *et al*., 1984; Robinson, 1997). Biggs *et al*. (1983) and Enebak *et al*. (1997) also observed an increased reactivity to histochemicals but reduced meristematic activity in inoculated plants in comparison with uninoculated wounds.

Despite the high disease pressure at 28°C, phosphite effectively restricted lesion extension in *E. marginata*. This result differs from the work of Swart and Denman (2000), where phosphite treatment was ineffective against the development of lesions caused by *P. cinnamomi* in *Leucospermum* hybrids under warm wet

conditions. However, the inoculation method and times of phosphite treatment and harvest were very different from the current study. At 28°C, the histochemical and anatomical responses of *E. marginata* inversely reflected the disease development, as has been reported in aspen (Enebak *et al*., 1997) and peach trees (Biggs and Miles, 1985; 1988). The most extensive responses were detected in plants with the least disease development at 28°C – the resistant clonal line treated with phosphite. The appearance of cracks at the lesion edge was an indication that the diseased tissue would eventually be shed, as seen in Chapters 4 and 5. The observation that maximum control of *P. cinnamomi* was achieved in the RR line supports the suggestion by Guest (1986) and Smillie *et al*. (1989) that host resistance influences the effectiveness of phosphite.

The low temperature used in the current study (20°C) favoured the host over the pathogen. Disease development was too restricted to show any effect of genotype or phosphite treatment on lesion extension. Histological studies of the interaction at 20°C revealed that both the resistant and susceptible clonal lines responded to the presence of *P. cinnamomi*. In contrast with the results of Chapter 5, suberin was detected at the lesion edge in plants of the SS line. The slow extension of *P. cinnamomi* gave normally susceptible plants enough time to respond. However, it is uncertain how durable or effective the barrier would be in the longer term. Hüberli *et al*. (2002) reported an apparent resistance of the susceptible clonal line to *P. cinnamomi* at 20°C until 2 weeks after inoculation, when plants started to die. This demonstrates the limitations of short-term experiments when examining host-pathogen interactions.

The inability to detect suberin in extant cells in wounded but uninoculated *E. marginata* plants in Chapter 5 and the current study agrees with the observations of Mullick (1977) and Soo (1977) but differs from the findings of Biggs (1984a; 1985a; 1985b), Rittinger *et al*. (1987) and Woodward and Pearce (1988), despite the

use of the same combination of histochemical tests and fluorescence microscopy used in the 1980s. Hawkins and Boudet (1996) also detected suberin in extant cells of *Eucalyptus gunnii* in response to wounding. It is possible that interference by phenolics (as reported by Woodward and Pearce, 1988) or the thickness of hand sections prevented the detection of any suberin present. The observed dome-shaped suberised cells with lignified outer cell walls, which were interpreted as the first phellem of the periderm, were very similar in appearance to cells considered by Robinson (1997) to be extant cells caught between the non-suberised impervious tissue (NIT) and the developing phellogen. Evidence to suggest the cells were phellogen derivatives in *E. marginata* was the radial alignment of these cells with the phellogen and their similarity to descriptions of first phellem in peaches by Biggs (1984b) and in *E. marginata* by O'Gara (1998). The cubical phellem cells discussed in Chapter 5 were observed again in radial alignment with the first-formed phellem. The current study was not able to clarify the origin of these cells.

The similarity of lesion lengths in the two clonal lines at 24°C differs from the results of Cahill *et al*. (1993), who reported significantly greater lesions in clonal line 11J402 than in 1J30 at 24°C. Cahill *et al*. (1993) inoculated roots with zoospores rather than stems with mycelium, and this difference in methods may have contributed to the contrast in results. Recently, Hüberli *et al*. (2002) determined that the expression of resistance by *E. marginata* clonal lines was influenced by the inoculation method. The high inoculum load in the current study may have tipped the balance in favour of the pathogen over the host at 24°C, overriding the stronger histological response in the RR clonal line. Incubating the stems at 23°C rather than 24°C gave a better distinction in lesion development between the two clonal lines.

In Chapter 5, it was reported that the extension of *P. cinnamomi* beyond the lesion was greater in the RR than in the SS clonal line. This was seen again in the current study. Although necrosis was occasionally observed in the inner bark and

xylem up to 1 cm beyond the measured lesion edge, it was not observed often enough to account for the discrepancies between the recorded lesion length and colonisation by the pathogen. Since only one half portion of each stem was examined histologically, it is possible that the plated stem segments were both colonised and lesioned internally. Alternatively, *P. cinnamomi* may be behaving as a hemibiotroph in the RR clonal line, as suggested in previous chapters, extending through the plants without causing any immediate obvious damage. Scraping the stems to expose any internal lesions, as done by Denman and Sadie (2001), would possibly have assisted in determining lesion lengths more accurately but would have also destroyed the histological material.

In contrast with Chapter 5, stem girdling did not always result in the death of plants. This was also observed by Hüberli *et al*. (2002) in an experiment conducted in controlled environment cabinets, and differs from reports that plants die as a result of stem girdling by *P. cinnamomi* (Shea, 1979; Marks and Smith, 1981; Marks *et al*., 1981; McCredie *et al*., 1985). The environment in the cabinets is different from the usual temperature and humidity fluctuations experienced by plants grown under more natural conditions, and may have altered the behaviour and effects of *P. cinnamomi* in *E. marginata*.

These results show that the outcome of the *E. marginata*-*P. cinnamomi* interaction is influenced by temperature, plant genotype and phosphite treatment. At temperatures optimum for disease development, phosphite is capable of protecting young plants of the resistant clonal line of *E. marginata* from the disease caused by *P. cinnamomi* when it is applied at two days after stem inoculation. At low temperatures, *E. marginata* is capable of defending itself against *P. cinnamomi* in the short term.

Chapter 7: General discussion

7.1 Introduction

This is the first study to examine the effects of phosphite on the histological responses of *Eucalyptus marginata* to *P. cinnamomi* and abiotic wounding. It is an extension of the work presented in Tippett *et al.* (1983), Tippett and Hill (1984) and Tippett *et al.* (1985), which discuss the importance of wound periderms in the resistance of *E. marginata* to *P. cinnamomi* in the absence of chemical treatment. In the present study, the combination of histological studies and assessment of disease development after phosphite treatment has provided a greater understanding of the role of phosphite in the protection of *E. marginata* from colonisation by *P. cinnamomi*.

7.2 Is phosphite effective against *P. cinnamomi* in *E. marginata*?

The work described in this thesis demonstrated that phosphite has the potential to enhance the resistance of young *E. marginata* and enable them to survive when infected by *P. cinnamomi*. However, the effectiveness of phosphite is influenced by a number of factors, including host resistance, environmental conditions, the applied phosphite concentration and the timing of application in relation to infection. Under high disease pressure in the glasshouse, treatment with 2.5 g phosphite/L was ineffective at preventing the death of *E. marginata* clones. In the field, treatment with this phosphite concentration had mixed success at inhibiting *P. cinnamomi* in seedlings of *E. marginata*. The 5 g phosphite/L treatment produced similarly mixed results in the field, although a longer duration of effectiveness against lesion extension was observed in comparison with the 2.5 g phosphite/L treatment. In the glasshouse, spraying with 5 g phosphite/L was sufficient to protect the RR clonal line but not the SS clonal line. Treatment with 10 g phosphite/L protected the SS clonal line from *P. cinnamomi*, but was phytotoxic and ineffective in the RR plants,

and phytotoxic to seedlings in the field. It is recommended that *E. marginata* seedlings under threat from *P. cinnamomi* are sprayed with 5 g phosphite/L annually, which agrees with recommendations by Hardy *et al*. (2001) for phosphite application in natural ecosystems. This is the treatment considered most likely to be effective at protecting young *E. marginata* from *P. cinnamomi*.

7.3 Anatomical responses of *E. marginata* to *P. cinnamomi* and wounding

Disease assessments in plants infected with *P. cinnamomi* commonly involve a quick and non-destructive measurement of lesion lengths. In this thesis, phosphite treatment restricted lesion extension in *E. marginata* stems. Histological examination of plants with a restricted extension of *P. cinnamomi* revealed a response similar to that described by Tippett *et al*. (1983) and Tippett and Hill (1984) – the development of a wound periderm at the junction of healthy and diseased tissue.

In the current study, periderm formation was not limited to plants treated with phosphite. This finding is not surprising since periderm formation is a common reaction of plants to both abiotic and biotic injuries (Mullick and Jensen, 1973). Untreated plants in the resistant clonal line and those incubated at temperatures sub-optimal for the growth of *P. cinnamomi* also formed periderms adjacent to necrotic tissue. The response of plants in the susceptible clonal line to wounding and to *P. cinnamomi* at 20°C supports the claim by Kuć (1983) that susceptible plants possess the machinery necessary for resistance.

The formation of a ligno-suberised boundary zone and wound periderm is a final stage in the series of events that occur following injury to a plant (Biggs, 1992) and is considered to be actively involved in defence (Biggs *et al*., 1984). However, the observed time delay of several days between lesion inhibition and the development of a ligno-suberised boundary zone and wound periderm in the current

study suggests that these slow responses did not play an active role in lesion inhibition when *E. marginata* was under high disease pressure. Pearce (1987) proposed that early defence responses such as the production of phytoalexins slow down pathogen growth and allow time for the development of a wound periderm. This proposal suggests a more passive role for periderms, preventing further pathogen spread after other defences have slowed pathogen growth (Biggs *et al*., 1984). There is considerable evidence from studies of other host-pathogen interactions that infected plants treated with phosphite or Fosetyl-Al accumulate high concentrations of early defence compounds such as phytoalexins (Guest, 1984; Khan *et al*., 1986; Saindrenan *et al*., 1988a; Afek and Sztejnberg, 1989; Nemestothy and Guest, 1990). When phosphite successfully slowed down pathogen growth in the current study, either by its direct action on *P. cinnamomi* or by an indirect stimulation of early defences, it provided *E. marginata* with the time and opportunity to form a wound periderm.

Since *P. cinnamomi* remains alive in phosphite-treated plants (Marks and Smith, 1992; Pilbeam *et al*., 2000; Wilkinson *et al*., 2001b), it is important for infected plants to form durable barriers to impede any further advances of the pathogen – their survival depends on it (Shigo, 1984). Wound periderms are considered to function as barriers to the extension of pathogens (Biggs, 1992). But is the wound periderm a durable barrier to the extension of *P. cinnamomi* in *E. marginata*?

7.4 Effectiveness of wound periderm against *P. cinnamomi* in *E. marginata*

Tippett *et al*. (1985) noted that the susceptibility of *E. marginata* to *P. cinnamomi* was associated with the host's inability to prevent renewed advance of the pathogen from lesions that may have been confined initially. In the current study, there were occasions when lesions appeared contained for several days prior to a sudden rapid

increase in the lesion length. This, combined with the high frequency of *P. cinnamomi* isolation beyond symptomatic tissue, suggests that it was the lesion rather than the pathogen that was contained temporarily. Lesions only become evident after cellular necrosis and lysis are sufficiently widespread throughout the tissue (Phillips, 1989). In the current study, it appeared that plants with a restricted lesion extension (including phosphite-treated plants) prevented the growth of an extensive front of *P. cinnamomi* through the bark but were generally unable to stop hyphal growth completely. As a consequence there was a delay in the disease development rather than complete control of *P. cinnamomi*, which is characteristic of horizontal resistance (Erwin and Ribeiro, 1996).

Given the difficulty in observing hyphae of *P. cinnamomi* in histological sections, it is not possible to determine whether the pathogen penetrated suberised cells of the wound periderm directly or circumvented the periderm. A reliable technique to observe hyphae of *P. cinnamomi in planta* needs to be developed before this can be investigated thoroughly. The observations of O'Gara (1998) suggest that *P. cinnamomi* uses both methods during the infection process, invading *E. marginata* through the thin-walled phellem directly and through discontinuities in the normal periderm. The periderms observed in the current study were generally incomplete. According to Biggs (1992), most hosts form a series of incomplete periderms rather than having the ability to stop pathogen invasion with a single wound periderm.

Since damage caused by *P. cinnamomi* extended into the vascular cambium, it is possible that *P. cinnamomi* circumvented bark defences by growing through the xylem. Davison *et al*. (1994) demonstrated xylem invasion by the pathogen in *E. marginata*, and suggested that this may explain sudden increases in bark lesions. Unfortunately phosphite did not limit invasion by *P. cinnamomi* to cortical tissues as observed in *Leucadendron* hybrids by Marks and Smith (1992).

Although samples were not taken to assess the extent of damage at the time of phosphite treatment, it is likely that vascular cambium damage had already occurred. The wound-inoculation technique inserted a large inoculum load deep into the phloem of the young *E. marginata*. Depth of inoculation has been shown to affect disease development in *Leucospermum* (Denman and Sadie, 2001). Although the wound-inoculation technique has been utilised in several studies of host-*P. cinnamomi* interactions (McCredie *et al*., 1985; Tippett *et al*., 1985; Shearer *et al*., 1988; Smith *et al*., 1997) and in most anatomical studies of canker fungi (Biggs, 1992), it is unrealistic and can suppress or delay host responses (Merrill and Shigo, 1979; Matta, 1981). It is recommended that more natural methods of inoculation be utilised in future research on host-*P. cinnamomi* interactions. The non-invasive technique described in Lucas *et al*. (2002) may provide an efficient and effective alternative to wound-inoculation.

7.5 Resistance of the resistant clonal line

Clonal lines with varying degrees of resistance to *P. cinnamomi* are a very useful resource for studying the effect of phosphite on the host-*P. cinnamomi* interaction. However, in contrast to the previously reported resistance of the RR clonal line to *P. cinnamomi* in controlled environment cabinets (Cahill *et al*., 1993) and in the forest (McComb *et al*., 1990; Colquhoun, personal communication), there was no consistent reduction of disease development in the RR plants in comparison with the SS clonal line in the current study. A combination of inoculation technique and environmental conditions is likely to have resulted in too high a disease pressure for a full expression of resistance in the RR clonal line. This highlights that phosphite was generally tested under high disease pressure in the clonal plants. In order to gain maximum benefits from using clonal lines, further phosphite research using the

clonal plants should be conducted under conditions that enable the full expression of resistance in the RR clonal line.

7.6 Conclusion

To ensure their survival, plants need to respond to invasion by a pathogen before too much damage is inflicted. Similarly, the pathogen endeavours to cause as much damage as possible before the plant can respond (Shigo, 1984). *E. marginata* has the capacity to respond to infection by *P. cinnamomi*, but the response may not be quick or strong enough to inhibit the pathogen. Phosphite is a tool with the potential to thwart the progress of *P. cinnamomi* and give *E. marginata* the opportunity to respond before extensive damage occurs.

Appendix 1: List of abbreviations

ANCOVA	analysis of covariance
ANOVA	analysis of variance
AW	after wounding
B	bark
Ca	callus
CALM	Department of Conservation and Land Management
Co	cortex
CODIT	compartmentalisation of decay in trees
CZ	cambial zone
EC_{50}	effective concentration 50
EP	exophylactic periderm
ES	empty space
EX	extant xylem
F-F	ferric chloride-potassium ferricyanide
FEP	first exophylactic periderm
HCl	hydrochloric acid
HP	healthy phloem
HPIC	high-performance ion chromatography
HT	healthy tissue
IP	inoculation point
IUPAC	International Union of Pure and Applied Chemistry
K	kino vein
LT	lesioned tissue
LX	lesioned xylem
MANCOVA	multivariate analysis of covariance
MANOVA	multivariate analysis of variance
NARPH	nystatin ampicillin rifampicin PCNB hymexazol
NeP	necrotic phloem
NIT	non-suberised impervious tissue
NmP	normal periderm
NP	necrophylactic periderm
NPF	new phloem fibres
NT	not tested
NX	new xylem
OVC	original vascular cambium
P	phloem
Pd	phelloderm
½PDA	half strength potato dextrose agar
PF	phloem fibres
Pg	phellogen
Ph	phellem
PHCl	Phloroglucinol HCl
PP	polyphenolic
PPF	primary phloem fibre
SB	sloughed bark
SBB	Sudan Black B
SD	suberin deposits
SE	standard error
SEP	sequent exophylactic periderm
SIT	suberised impervious tissue
SP	secondary phloem
SS	susceptible
RR	resistant
TkP	thick-walled phellem
TnP	thin-walled phellem
TP	traumatic parenchyma
VC	vascular cambium
WP	wound periderm
WT	wounded tissue
WW	woundwood
X	xylem
XPP	xylem pressure potential

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